GUANIDINES AND PHOSPHAGENS OF SEMEN

by I. G. WHITE AND D. E. GRIFFITHS[1]

(From the Department of Veterinary Physiology, University of Sydney, and the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra).

(*Accepted for publication 2nd December, 1957.*)

The first report of the occurrence of guanidines in reproductive organs was made by Treskin (1872) who showed that creatine and phosphocreatine were present in the testes of the bull. These compounds have been shown to occur also in bull, stallion and boar semen (Ilyasov, 1933; McKenzie, Miller and Bauguess, 1938). Eggleton and Eggleton (1929) showed that phosphocreatine was present in animal testes and suggested that the phosphagen might also occur in spermatozoa. It may be noted in this connection that Torres (1935) claimed that phosphocreatine was synthesised from creatine and phosphopyruvate by bovine epididymal spermatozoa, but this claim was refuted by Ivanov (1937). More recently Wajzer and Brochart (1947) reported the presence of phosphoarginine as well as phosphocreatine in boar and guinea pig semen. This finding is of interest because of the generally held view that phosphocreatine is the characteristic phosphagen of vertebrate tissue.

The present study was designed to determine the distribution of seminal phosphagens and guanidines in a number of species.

MATERIALS AND METHODS.

The following abbreviations will be used: PC—phosphocreatine; PA—phosphoarginine; CPK—creatine phosphokinase; APK—arginine phosphokinase; ADP—adenosine diphosphate.

Collection and Fractionation of Semen. Bull and rabbit semen was collected by the artificial vagina (Walton, 1945; White, 1955), ram semen by electrical ejaculation (Blackshaw, 1954), and human, dog and fowl (Burrows and Quinn, 1939) semen by massage. After collection, the semen was pooled and stored at $-79°$ C. or $-30°$ C. The time between collection and freezing of the semen was usually less than 1 hr. with the exception of human semen which was not frozen until 5 hr. after collection. In all cases, the spermatozoa showed good motility at the time of freezing. The final material for analysis consisted of more than 20 pooled ejaculates from each species except the dog, where 3 pooled semen samples and 5 pooled specimens of accessory secretion were used.

Spermatozoa were separated from the seminal plasma following thawing by centrifugation at 1,000-5,000 r.p.m. for 15-30 min. They were then washed twice with 3 vol. of 0·9 p.c. (w/v) NaCl.

---

[1] Australian National University Research Scholar.

Extraction of Guanidines. Because of the large amount of polypeptides and low molecular weight proteins present in semen (Mann, 1954) ethanol was used to precipitate these compounds and at the same time extract the guanidines. Seminal plasma was extracted by treating with 6 vol. of 80 p.c. (v/v) ethanol. The mixture was boiled for 5 min. and centrifuged. The supernatant was allowed to stand overnight at 5° C. and any precipitate which formed was removed by centrifugation. If, after the above treatment, the supernatant was opalescent (as in the case of ram seminal plasma), it was clarified by the further addition of 80 p.c. (v/v) ethanol, followed by centrifugation. Extracts of spermatozoa were prepared by grinding the washed cells with acid-washed powdered glass in the presence of 6 vol. 80 p.c. (v/v) ethanol, followed by centrifugation. Because of the low guanidine content of spermatozoa, it was necessary to concentrate the ethanolic extracts prior to estimation.

Estimation of Guanidines. The guanidine content of the above extracts and of untreated seminal plasma was estimated by the colorimetric method of Rosenberg, Ennor and Morrison (1956). Equivalent amounts of ethanol were added to the standards to compensate for the effect of the ethanol present in the extracts on the intensity of the colour developed.

Characterisation of Guanidines. Ethanol extracts were taken to dryness on a boiling water bath, the residue dissolved in water and the solution clarified by centrifugation. Paper chromatographic examination was then carried out by the ascending method at room temperature using sheets of Whatman No. 3 MM paper (12 in. x 12 in.). Chromatograms were developed in the solvents described by Griffiths, Morrison and Ennor (1957).

The guanidines present in semen extracts were also characterised by enzymic means. Arginase, CPK and APK were used under the conditions described by Griffiths *et al.* (1957).

Isolation of Phosphagens. Ram, bull and rabbit spermatozoa and guinea pig testes were extracted with 4 vol. of ice-cold 9 p.c. (w/v) trichloracetic acid by treatment in a Waring blendor for 2 min. The mixture was filtered on a Buchner funnel and the filtrate adjusted to pH 9 with 10 N NaOH. To seminal plasma was added trichloracetic acid (100 p.c., w/v) to a concentration of 10 p.c. and the mixture was treated as described above. Phosphagens were isolated as Ba salts and converted to Na salts as described by Griffiths *et al.* (1957).

RESULTS.

Seminal Plasma. For comparative purposes, analyses of the guanidine content were carried out on the untreated seminal plasma and on ethanolic extracts. In both cases (Table 1) there was considerable variation in the total guanidine content of samples from different animals. Also, with the exception of the fowl and human seminal plasma, the guanidine content of ethanolic extracts was very much less than that of the untreated seminal plasma.

The higher guanidine values for untreated seminal plasma were due to the reaction of the bound arginine of polypeptides and proteins with the diacetyl-α-naphthol reagent. Thus analysis of untreated ram seminal plasma by means of arginase gave an arginine value of 21 mg. p.c. which is in good agreement with the values of 17 and 21 mg. p.c. obtained by analysis of the ethanolic extract using arginase and APK respectively (Table 1).

Chromatographic and enzymic examination showed that arginine and creatine were present in varying proportions in the seminal plasma of all animals except the dog in which arginine was the only guanidine identified. Creatine was found, however, in a sample of dog accessory secretion. Chromatography

TABLE 1.

Distribution of guanidines in seminal plasma.

Species	Total guanidine content mg. p.c. (expressed as arginine)		Paper chromatography of ethanol extracts		Concn. of guanidines in ethanol extracts mg. p.c.			
	Seminal plasma	Ethanol extract of plasma	No. of guanidines detected	Guanidines identified	Arginine		Creatine	Unidentified guanidines expressed as arginine
					a	b	c	
Bull	99	18	4	Arginine				
				Creatine	1	—	—	—
Dog	37	6	1	Arginine	4	2	0	—
Fowl	107	95	2	Arginine				
				Creatine	18	—	41	—
Human	134	153	4	Arginine				
				Creatine	91	87	17	44
Rabbit	40	10	2	Arginine				
				Creatine	1	—	2	0
Ram	58	27	2	Arginine				
				Creatine	17	21	2	0

(a) These values represent the difference in the guanidine content of samples of the extract before and after treatment with arginase.

(b) and (c) These values were calculated from the disappearance of guanidine following incubation with either CPK or APK. Allowance was made for the fact that at equilibrium only 90 p.c. of arginine or creatine is phosphorylated.

of human and bull seminal plasma extracts revealed the presence of two unidentified guanidines. The quantity of material available did not permit of further identification, but all were mono-substituted guanidines as judged by the Sakaguchi reaction.

No evidence was obtained for the presence of phosphagens or N-amidinophosphokinases in seminal plasma. Thus hydrolysis of ethanolic extracts in 0·1N HCl for 1 min. at 100° C. did not increase the free guanidine content; also attempts to isolate phosphagens from ram and bull seminal plasma by the barium precipitation procedure led to completely negative results. The presence of APK or CPK could not be demonstrated; there was no release of guanidine from PA or PC when samples of seminal plasma were incubated in the presence of ADP under the appropriate conditions.

Spermatozoa. The guanidine content of ethanolic extracts of washed spermatozoa was small (bull, 6 mg. p.c.; rabbit and ram, 4 mg. p.p.) and attempts to demonstrate the presence of phosphagens by isolation and enzymic techniques were unsuccessful. No CPK or APK activity was found in aqueous extracts of ram, rabbit, bull or fowl spermatozoa.

Guinea Pig Testes. In view of the results of Wajzer and Brochart (1947), analyses were made of the guanidine and phosphagen contents of guinea pig

testes and aqueous extracts were tested for CPK and APK activity. Creatine and PC only were found to be present; the concentration of creatine was 33 mg. p.c. whilst the concentration of PC varied between 10-25 mg. p.c. CPK activity was readily demonstrated, but there was no evidence for the presence of APK.

DISCUSSION.

As the diacetyl-α-naphthol reagent used for the estimation of guanidines also reacts with the bound arginine of proteins and polypeptides, the guanidine values obtained by analysis of untreated seminal plasma may not be a true measure of the guanidine content. On the other hand, as both proteins and polypeptides are precipitated by boiling ethanol, analysis of ethanolic extracts of the plasma should give correct values for the free guanidine content. This point is well illustrated by comparison of the results obtained with bull, dog, rabbit and ram seminal plasma where the values for the untreated plasma are very much higher than those of the ethanolic extracts. The agreement of the results obtained by the use of arginase on untreated seminal plasma and on ethanolic extracts also indicates that values obtained for the guanidine content of untreated seminal plasma estimated by the diacetyl-α-naphthol reagent would include those values due to the N-amidino-group of polypeptides and proteins. Extracts which are opalescent owing to the presence of polypeptides or protein also give higher values.

There would seem to be marked differences in both the arginine and the creatine content of the semen of the various species, although further analyses of individual ejaculates are necessary to establish this point fully. While the values for creatine are undoubtedly correct, it is possible that the arginine values are high because of proteolytic enzyme activity during the interval between collection and freezing, particularly with the human semen. It is of interest that guanidines, other than arginine and creatine, are present in bull and human seminal plasma.

Failure to demonstrate the presence of phosphagens or N-amidinophosphokinases in either spermatozoa or seminal plasma suggests that the motility of spermatozoa may not be concerned with transphosphorylation reactions involving phosphagens. These results, together with failure to demonstrate APK activity in extracts of guinea pig testes, cast doubt on the claim of Wajzer and Brochart (1947) that PC and PA are present in guinea pig and boar spermatozoa. The presence of phosphagen in the testes of the guinea pig and other vertebrates (Ennor and Rosenberg, 1952), as well as in the gonads of invertebrates (Griffiths, Morrison and Ennor, 1957), suggests that phosphagens do play a rôle in the general reactions of reproductive organs.

SUMMARY.

The distribution of guanidines in bull, dog, fowl, human, rabbit and ram seminal plasma and spermatozoa has been determined.

No evidence was obtained for the presence of phosphagens or N-amidinophosphokinases in the seminal plasma or spermatozoa of these animals.

Acknowledgment. Our thanks are due to Professor C. W. Emmens, Professor A. H. Ennor, and Dr. J. F. Morrison for their interest and advice, and to Miss T. Kaak for skilled technical assistance.

REFERENCES.

Blackshaw, A. W. (1954): Austral. Vet. J., 26, p. 249.
Burrows, W. H., and Quinn, J. P. (1939): U.S. Dept. Agr. Circ. 525. Artificial Insemination of Chickens and Turkeys.
Eggleton, G. P., and Eggleton, P. (1929): J. Physiol., 68, p. 193.
Ennor, A. H., and Rosenberg, H. B. (1952): Biochem. J., 51, p. 606.
Griffiths, D. E., Morrison, J. F., and Ennor, A. H. (1957): *Ibid.*, 65, p. 612.
Ilyasov, I. (1933): Trans. Vet. Path., Orenburg Vet. Inst., 48, c.f. Anim. Breed. Abstr., 3, p. 479.
Ivanov, I. I. (1937): Biokhimiya, 2, p. 926; Chem. Abstr., 32, p. 2584.
Mann, T. (1954): The Biochemistry of Semen, London: Methuen.
McKenzie, F. F., Miller, J. C., and Bauguess, L. C. (1938): Res. Bull. Mo. Agric. Exp. Sta., p. 279.
Rosenberg, H., Ennor, A. H., and Morrison, J. F. (1956): Biochem. J., 63, p. 153.
Torres, I. (1935): Biochem. Z., 283, p. 128.
Treskin (1872): Pflug. Arch. ges. Physiol., 5, p. 122.
Walton, A. (1945): Notes on Artificial Insemination of Sheep, Cattle and Horses. London: Holborn Surgical Instrument Co.
Wajzer, J., and Brochart, M. (1947): C.R. Acad. Sci., Paris, 225, p. 965.
White, I. G. (1955): Austral. J. exp. Biol., 33, p. 267.

INFERTILITY ASSOCIATED WITH EXTIRPATION OF THE OLFACTORY BULBS IN FEMALE ALBINO MICE

By D. R. LAMOND

(From the Department of Veterinary Physiology, University of Sydney, Sydney, Australia).

(*Accepted for publication 21st November, 1957.*)

Complete removal of the olfactory bulbs of adult female mice has been shown to bring about a significant reduction in weight of the uteri and ovaries over a six weeks' period (Whitten, 1956b). It was further observed that mating failed to take place for at least 12 days after the operation. These and related findings (see Whitten, 1956a) strongly suggest that exteroceptive stimuli involving the olfactory pathways may affect reproduction. Some aspects of the problem have been recently investigated in this laboratory and the findings indicate its complex nature.

Materials and Methods.

Immature randomly bred female albino mice were weaned at 18 days, by which time body weight was approximately 10 gm. Three days after weaning the mice were allotted at random to cages, each containing five animals. They were housed in a room heated to 76 ± 2 deg. F. with 14 air changes per hour. The daily light:dark ratio was kept constant at 12:12 hr. All observations were carried out in daylight.

Body weights were recorded at weaning and weekly thereafter. Vaginal smears were taken six days each week, using a metal spatula. A positive smear was characterised by nucleated epithelial or squamous cells in the absence of leucocytes.

The olfactory pathway was interrupted by a modification of the method of Whitten (1956b). The bulbs were removed by suction under ether anaesthesia after clearing the cribriform plate connections. The olfactory tracts were not severed and this undoubtedly accounts for the high proportion of mice in which there remained small portions of bulb.

Description of the treatment groups is summarised in Table 1. All mice were weaned on the same date. They were killed when approximately 155 days old, when ovarian weights were recorded and the heads placed in formalin. Examination of the olfactory region was carried out using a binocular dissecting microscope and the mice were graded according to whether the bulbs had been completely removed (A), or whether portions of the bulbs remained (B). Controls consisted of groups of similar animals which were housed with males prior to puberty, two weeks after puberty or after three months of vaginal smearing.

TABLE 1.

Description of treatment groups. All mice were killed at 155 days.

Group	Age of mice in days				
	21	30	65	78	125
1	Removal of olfactory bulbs	One male placed in each cage		Males replaced	Paired monogamously with males
2	Removal of olfactory bulbs	Commencement of vaginal smears			Paired monogamously with males
3		Commencement of vaginal smears	Removal of olfactory bulbs		Paired monogamously with males
4		Commencement of vaginal smears	Removal of olfactory bulbs	One male placed in each cage	Paired monogamously with males

RESULTS.

Success of operation.

Of 75 mice which survived to 155 days, 28 (37 p.c.) were assumed to be completely anosmic since only portions of the olfactory tracts remained. There was apparently greater success with mice which had been operated after puberty

TABLE 2.

Summary of breeding history at completion of experiment.

Parous mice had given birth to at least one normal litter or were pregnant. Ovarian weights ±S.E. have been included for each sub-group.

Group	A—bulbs completely removed		B—bulbs incompletely removed		Total parous mice	No. survivors
	Parous	Non-parous	Parous	Non-parous		
1	2 13·0±0·5	2 6·0±1·0	9 10·8±0·7	6 5·4±0·4	11	19
2	2 13·0±2·0	5 5·4±0·7	4 10·0±0·8	6 6·0±0·7	6	17
3	1 9·5	8 6·2±0·7	5 11·6±0·8	5 6·8±0·9	6	19
4	4 11·9±1·0	4 7·1±1·3	7 11·9±0·9	5 5·6±0·4	11	20

(Groups 3 and 4, Table 2). The remaining mice (B, Table 2) had small portions of bulbs attached to the olfactory tracts. In each of five mice there was some connection to the cribriform plate. It is most likely, therefore, that the majority of mice were completely anosmic.

The importance of classifying the data as in Table 2 lies in the demonstration that a total of 9 mice, about which there was no doubt of interruption of the olfactory pathway and complete extirpation of the bulbs, gave birth to normal litters during the course of the experiment. In addition, there was no significant difference in the proportion of parous mice in the two classified groups $X^2_{(1)} = 3{\cdot}14$; $0{\cdot}05 < P < 0{\cdot}1$. The combined data show that 34 (45·3 p.c.) mice had given birth to normal litters or were pregnant by 155 days. All of 79 control mice had given birth to litters by this time.

Effect of treatments.

There was a significant difference in the total number of parous mice between the groups which were housed with males throughout (Groups 1 and 4, Table 2) and the groups which were mated only for the final 30 days (Groups 2 and 3), $X^2_{(1)} = 4{\cdot}02$; $0{\cdot}02 < P < 0{\cdot}05$.

The number of mice which littered during successive periods after the operation is shown in Table 3. It can be seen that the similarity between groups 1 and 4 extends throughout the experimental period. The ablation of the olfactory bulbs, partial or complete, had no obvious immediate effect on a small proportion of mice. A further proportion later gave birth to normal litters so

TABLE 3.

Temporal changes in reproductive phenomena.

Group	Mice littering during period:				Total parous mice
	to 95 days	95-115	115-135	135-155*	
1	2	3	4	8	11
4	0	3	4	9	11
	Mice experiencing oestrous cycles (and total cycles) during the period:				Conceptions during the period 125-155 days
	to 65 days	65-85	85-105	105-125	
2	8(12)	5(7)	10(12)	10(22)	6
3	19(38)	17(28)	14(27)	12(29)	6

* includes unborn litters.

that three months after puberty 22 out of 39 mice were similar to control animals. These results agree reasonably well with previous unpublished work in which there was a progressive increase in parous mice over a five month period so that a total of 22 mice out of 38 had given birth to at least one litter or were pregnant.

The occurrence of oestrous cycles in groups 2 and 3 is summarised in Table 3. By 105 days 10 mice in group 2 had experienced spasmodic cycles. In the period 105-125 days, however, the rate of cycling increased and this was reflected by six mice conceiving in the next 30 days. The results of group 3 clearly show that a similar proportion of mice were fertile. Considering the combined data it can be seen that of 22 mice apparently normal in the period 105-125 days only 12 conceived in the next 30 days.

The relationship between oestrus and fertility is shown in Table 3. In the early stages after the operation in the immature mice there is poor agreement between the number of mice which came into oestrus and those which conceived. When group 1 is corrected for the length of the gestation period ($19 \cdot 8 \pm 1 \cdot 0$ days in control animals) there is obviously a much better agreement at four months of age, which is associated with more regular cycling. Although not strictly comparable there is a similar trend in the comparison between groups 3 and 4. In addition, however, it can be seen from group 3 that two mice ceased cycling immediately after the operation, a further three had ceased within 20 days and two more in the next 20 days.

Influence of body weight.

The decreased rate of growth resulting from removal of the olfactory bulbs at 21 days apparently only persisted to the approximate time of puberty (Table 4). In the normal animals the first oestrus occurred at $20 \cdot 7 \pm 0 \cdot 2$ gm. (38 mice) body weight and was independent of age. Hence the net effect of the operation on reproductive performance was to delay the time at which the mice might be expected to reach puberty.

TABLE 4.

Body weights of mice operated before and after puberty.

The body weights of groups 1 and 2 and of groups 3 and 4 were similar and have been pooled to save space. The combined data for groups 3 and 4 are similar to control values.

Groups	Number of mice	Body weights ± S.E.			
		20 days	45 days	65 days	90 days
1 and 2	36	$9 \cdot 1 \pm 0 \cdot 2$	$18 \cdot 7 \pm 0 \cdot 4$	$21 \cdot 0 \pm 0 \cdot 4$	$24 \cdot 4 \pm 0 \cdot 3$
3 and 4	39	$9 \cdot 9 \pm 0 \cdot 3$	$21 \cdot 2 \pm 0 \cdot 3$	$23 \cdot 8 \pm 0 \cdot 4$	$26 \cdot 8 \pm 0 \cdot 4$

DISCUSSION.

The important feature of these findings is the demonstration that the intact olfactory pathway is not indispensable for birth of a normal litter in a proportion of mice. Nevertheless, there is ample evidence that the operation, as carried out, was responsible for considerable disturbances in reproductive function which cannot be satisfactorily explained in terms of partial or complete ablation of the bulbs.

Of 17 mice which were operated as immatures 8 experienced their first oestrous cycle at the expected time, when they reached approximately 21 gm. body weight. Subsequent cycling was, however, most irregular until the mice were three to four months of age. Although vaginal oestrus was well-defined at this time, the low proportion of mice which had become pregnant by five months suggests that either psychic oestrus was altered or there was some abnormality in the tract. The former possibility is the more likely since only five copulation plugs were observed and these were in mice which subsequently proved pregnant.

It is apparent that a similar situation holds for the mice operated on after puberty. There was a gradual decline in the number which experienced cycles until at 3-4 months of age 12 of 19 mice were showing vaginal oestrous cycles. Again, however, only half became pregnant and since copulation plugs were not observed in mice other than those which became pregnant it may be assumed that psychic oestrus was absent or reduced.

In order to accept the hypothesis of reduced or absent psychic oestrus it is necessary to explain the effect of the presence of the male as an exteroceptive stimulus in terms of pathways other than olfaction. Furthermore, it would be necessary to assume that events after the operation involve two steps: cessation or disturbance of ovarian function, and then return to normal function accompanied, however, by abnormal oestrus. A more simple explanation seems to be that the operation altered psychic phenomena including oestrus, in such a way that the female would not allow mating until continued efforts on the part of the male were successful. An extension of this hypothesis is based on the observation that haematoma formed around the optic nerves between the optic foramen and the optic chiasma and hence vision may have been impaired for a variable time, so that, lacking a sense of smell and keen vision, the female may have been wary of other mice, particularly recently introduced males.

Although the effects of the operation are subject to considerable individual variation it seems clear that interference with olfaction can bring about a characteristic alteration in some reproductive phenomena. Hence, while some mice are apparently unaffected others experience spasmodic vaginal oestrous cycles for a variable period after which they may return to normal. A further proportion of mice can be expected to cease overt cycling for an indefinite period and

this is associated with a decline in ovarian weight. It is possible that some such mice would remain in a state of permanent anoestrus.

No explanation can be offered as to the mechanism by which the operation brought about interference with reproduction. The results, however, do reflect very pointedly the extreme variability which is likely to be encountered in investigations of this kind.

SUMMARY.

Partial and complete ablation of the olfactory bulbs of immature and mature female albino mice resulted in infertility in a proportion of mice. The individual response to the operation was highly variable, some mice being apparently unaffected whilst others were infertile for periods up to at least five months.

It was suggested that the operation altered psychic phenomena including overt oestrus and that this may partly account for the results.

Acknowledgments. Grateful acknowledgment is made to Professor C. W. Emmens, and also to Dr. W. K. Whitten, Medical School, Canberra, for criticism and advice. The work was made possible by a grant from the Commonwealth Bank of Australia and the Wool Research Trust Fund.

REFERENCES.

Whitten, W. K. (1956a): J. Endocrinol., 13, p. 399.
Whitten, W. K. (1956b): *Ibid.*, 14, p. 160.

THE EFFECTS OF HYPERCHOLESTEROLAEMIA ON ARTERIAL GROUND SUBSTANCE IN RABBIT ATHEROSCLEROSIS

by COLIN J. SCHWARTZ,[1] J. ANDREW PETERS AND ALLAN J. DAY

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication 27th November, 1957.*)

There is abundant evidence that tissue damage predisposes to arterial lipid deposition and to atherogenesis (Schlichter *et al.*, 1949; Kelly *et al.*, 1952; Oester *et al.*, 1955; and Prior and Hutter, 1955). However, the mechanism by which this damage enhances atherogenesis is uncertain. Alterations in the histochemical staining of arterial mucopolysaccharides are the earliest demonstrable features of human atherosclerotic lesions (Taylor, 1953) and precede the deposition of lipids. Faber (1949) suggested that the mucopolysaccharides of mesenchyme may be directly concerned with cholesterol deposition in tissues including the aorta. Altshuler and Angevine (1951) suggested that lipid deposition and its tissue fixation may occur by a process of ionic exchange between circulating cations and partially depolymerized chondroitin sulphuric acid. If depolymerization of ground substance is a prerequisite for lipid deposition in arteries, then experimental depolymerization should result in enhanced atherogenesis. This has been adequately demonstrated in cholesterol-fed rabbits treated with testicular hyaluronidase (Benditt *et al.*, 1950; Seifter *et al.*, 1953; and Wang *et al.*, 1955). However, the possibility that hypercholesterolaemia may itself induce a breakdown of connective tissue mucopolysaccharides has not been explored. In this paper the possible rôle of connective tissue mucopolysaccharide breakdown in the pathogenesis of atherosclerosis in hypercholesterolaemic rabbits is investigated, together with the effect of hyaluronidase upon atherogenesis.

METHODS.

Twenty rabbits of mongrel stock, approximately six months of age (1·7 to 2·7 kg.) were used. All animals were fed a daily diet consisting of 75 gm. of mash, 1 gm. of cholesterol and 6 ml. of peanut oil for a total period of six weeks. After two weeks of

[1] This work was aided by a grant from the National Health and Medical Research Council of Australia.

cholesterol feeding they were randomly divided into two groups according to weight, sex, serum lipid and hexosamine levels. At this point the test group received a daily intramuscular injection of testicular hyaluronidase containing 750 Turbidity Reducing Units, while the control animals received an equivalent injection of normal saline. The rabbits were weighed weekly throughout the experiment.

Serum lipid and serum hexosamine levels were determined at the commencement of the experiment, after 14 days of cholesterol feeding and then after 3, 20 and 28 days of hyaluronidase or saline respectively.

Serum lipids. Serum cholesterol was determined by the method of Zlatkis, Zak and Boyle (1953) and serum phospholipid by the method of Brown (1954). In the case of both fractions a 3:1 ethanol-ether extract was used as previously described (Day and Peters, 1958).

Serum hexosamine levels were determined by the method of West and Clarke (1938) using 0·1 ml. of serum. The colour developed was read in a "Unicam" S.P. 600 spectrophotometer at 540 mμ. Serum hexosamine was expressed as mg./100 mls. of glucosamine using as a standard glucosamine hydrochloride.[2]

Aortic atheroma was graded macroscopically as described by Day and Wilkinson (1956).

Aortic valve atheroma was assessed on the cusps by direct observation of the fresh unstained specimens, the number of discrete lesions being noted in each animal.

Histopathology. All animals were killed by the intravenous injection of pentone. After removal of the aortae and the grading of aortic atheroma, the tissues were immediately fixed in 10 p.c. neutral formalin. Following fixation the aortae were stripped of adventitial debris and coiled longitudinally to include the whole length from the origin to the bifurcation. Multiple 6 μ paraffin sections were prepared. Similar sections of heart, heart valves, liver and kidney were also prepared. Haematoxylin and eosin; (H. & E.), Periodic acid Schiff (P.A.S.), Toluidine blue and Crossman stains were prepared on all sections.

RESULTS.

Atheroma. From Table 1 it can be seen that there was no significant difference in macroscopic aortic atheroma between the two groups, but the incidence and severity of macroscopic lesions on the aortic cusps has been greatly enhanced by hyaluronidase treatment. Within the test group eight of the ten rabbits had plaques on the aortic cusps and these lesions were considerably more extensive than the few found in the control group.

Serum lipids. The results of serum cholesterol and phospholipid determinations are not included as no significant differences between the two groups were noted.

Serum hexosamine. At the commencement of the experiment the mean serum hexosamine levels in both groups, test and control, were approximately the same, being 91·0 (± 2·48) and 95·0 (± 2·40) mg. per 100 ml. respectively. Following hyaluronidase treatment the serum hexosamine levels of the test group rose to a mean of 115 (± 2·40) mg. per 100 ml., 129 (± 1·63) mg. per 100 ml. and 138 (± 3·73) mg. per 100 ml. at 3, 20 and 28 days respectively (Table 2).

---

[2] D. glucosamine hydrochloride. Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.

TABLE 1.

The effect of hyaluronidase on the severity of atherosclerosis in cholesterol-fed rabbits.

Treatment	Rabbit	Aortic Atheroma Grade	Aortic valves Number of Lesions
	A1*	—	—
	A2	II	++++
	A3	I	++++
Test Animals	A4	II	++++
	A5	I	++
Cholesterol and	A6	I	0
hyaluronidase	A7	II	++++
	A8	0	++++
	A9	I	++
	A10	II	++++
	A11	II	0
	A12	III	0
Control Animals	A13	II	+
	A14	I	0
Cholesterol and saline	A15	I	0
	A16	I	+
	A17	I	+
	A18	I	+
	A19	I	0
	A20	I	+

* Excluded because of hepatic cirrhosis.

TABLE 2.

The effect of hyaluronidase and hypercholesterolaemia upon the mean serum hexosamine level in rabbits.

		Mean Serum Hexosamine (mg./100 ml.)			
	Initial level 0 days	14 days cholesterol feeding	17 days cholesterol 3 days hyaluronidase	34 days cholesterol 20 days hyaluronidase	42 days cholesterol 28 days hyaluronidase
Hyaluronidase	95·0(±2·40)	99·0(±1·77)	115·0(±2·40)	129·0(±1·63)	138·0(±3·73)
Control	91·0(±2·48)	97·0(±2·34)	98·0(±1·41)	113·0(±3·58)	112·0(±4·27)

The increase in mean hexosamine levels due to hypercholesterolaemia and to hyaluronidase treatment were both highly significant ($P < \cdot 01$ and $P < \cdot 001$ respectively).

Histopathology and Histochemistry.

With both H. and E. and Crossman stains, a diffuse hyaline fibrocellular thickening of the aortic intima was noted in both groups, control and test. This diffuse intimal thickening was decidedly more pronounced in the arch of the

Fig. 1. Rabbit aorta; cholesterol feeding. (H. & E. 120x.) The diffuse fibrocellular intimal thickening (I) is seen, merging with a foam cell plaque (F). The foam cells appear as vacuolated intimal cells arising *in situ*. Subjacent disorientation of the normal medial architecture is apparent.

Fig. 2. Rabbit aorta; hyaluronidase and cholesterol feeding (H. & E. 120x). Here a small section of the diffuse intimal lipoidosis (L) in the group treated with hyaluronidase is seen. The underlying media is again disorientated with an extensive elastolysis.

aorta than in the abdominal segment. At many points this thickening could be seen to merge with foam cell plaques (Fig. 1). The earliest stage of foam cell formation appeared as a vacuolation of the cells of the thickened intima. Foam cell cushions in the aorta were small and relatively infrequent in the control group. This finding contrasted sharply with the greater number and extent of foam cell plaques found in the test animals. In this latter group a diffuse intimal lipoidosis was commonly seen (Fig. 2). Foam cell plaques were also noted in the endocardium where they were particularly more extensive in the test group (Fig. 3). Well developed atheromatous lesions were seen in the aortae and coronary arteries of both groups. They consisted essentially of an extensive fibrocellular thickening of the intima and subintima, with a fragmentation, disruption and disorientation of the subjacent elastic laminae of the media. Lipid deposition in these lesions as foam cells was variable, but was more pronounced in the hyaluronidase group.

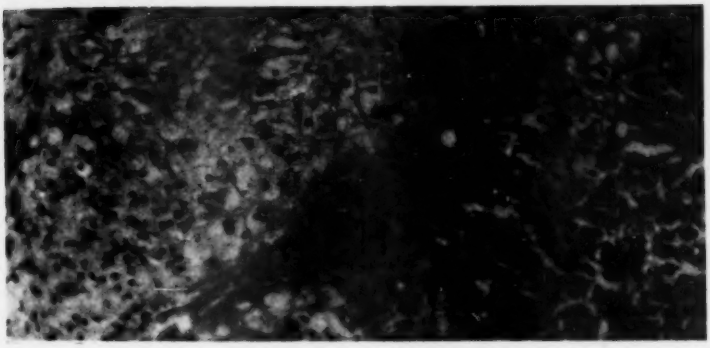

Fig. 3. Rabbit heart; hyaluronidase and cholesterol feeding (H. & E. 120x). An endocardial atheromatous lesion (A) lying upon the myocardium (M) is seen. In the intervening region is an area of intensely hyaline material (H) which is both P.A.S. positive and metachromatically staining.

Fig. 4. Rabbit aorta; cholesterol feeding (H. & E. 120x). An area of aortic cartilaginous metaplasia (C) is seen in this section. It is associated with marked metachromasia and P.A.S. positive staining.

In both control and test groups, several foci of cartilaginous metaplasia were noted (Fig. 4). Also seen in some animals of both groups were several areas of extensive medionecrosis.

In P.A.S. and toluidine blue treated sections within and subjacent to areas of intimal thickening, foam cell plaques and well developed atherosclerotic lesions, there were discrete and focal accumulations of metachromatic, P.A.S. positive material. There was no difference between groups in the intensity of the metachromasia and the amount of P.A.S. positive material, associated with atherosclerotic lesions. The mucopolysaccharide of lesions demonstrated in this way was therefore resistant to the *in vivo* action of testicular hyaluronidase A. Less intense metachromatic staining and a smaller amount of P.A.S. positive material was seen in the normal segments of aortae of both groups. The intensity of metachromatic staining and the presence of P.A.S. positive material were both greater in the thoracic end of the aorta than in the abdominal end.

Metachromasia was found particularly in the inner half of the media and in the subintima. There was less P.A.S. positive material present in the aortae of the test group than in the controls, but there was no appreciable difference between groups in the localization or intensity of metachromatic staining.

At the sites of cartilaginous metaplasia in the aortae, metachromatic staining was extremely intense as was the increase in P.A.S. positive material. The areas of medio-necrosis were likewise associated with a marked increase in metachromasia and P.A.S. positive material. These staining reactions corresponded to the brightly eosinophilic amorphous "fibrinoid" hyaline material observed in these lesions with the H. & E. stain. There was no difference observable in these staining reactions between the two groups. The lesions on the aortic valves were also associated with focal increases in metachromasia and P.A.S. positive material.

DISCUSSION.

An elevating effect of testicular hyaluronidase A on the serum hexosamine levels of hypercholesterolaemic rabbits has been demonstrated. This effect is due to the depolymerization and hydrolysis of the mucopolysaccharides in the ground substance of connective tissues. The principle mucopolysaccharides of significance are hyaluronic acid and the chondroitin sulphuric acid compounds. Chondroitin sulphate B (C.S.B.), chondroitin sulphate C (C.S.C.) and a dubious amount of hyaluronate are found in the aorta and heart valves (Meyer, 1954). Breakdown of these mucopolysaccharides both in the aorta and other sites should lead to an increase in the serum of their component amino sugars. That this is so has been demonstrated previously in rabbits (Schlamowitz *et al.*, 1950) and also in rats (Schwartz and Gilmore, 1958). Furthermore, the interpretation of the elevated hexosamine levels in the later stages of the hypercholesterolaemic control group can be more readily understood. It is reasonable to interpret this elevation as reflecting a breakdown or depolymerization of mesodermal ground substance mucopolysaccharides, induced by the high serum lipid levels, unless the hypercholesterolaemia has in some way influenced amino sugar metabolism itself. There is no evidence to support this latter contention. The findings of an elevation in serum amino sugars in hypercholesterolaemic rabbits is in agreement with the report of Antonini and Mininni (1955).

In further support of the concept of hypercholesterolaemia inducing mesenchymal ground substance breakdown are the findings of increased metachromasia and an accumulation of P.A.S. positive material at the sites of aortic atheroma plaques. These histochemical findings, together with the biochemical evidence of mucopolysaccharide depolymerization, suggest that mesodermal ground substance breakdown, as a sequel to hypercholesterolaemia, may be of significance in the pathogenesis of atherosclerosis in cholesterol-fed rabbits. Cortisone is known to inhibit collagen ground substance breakdown, and at the

same time to depress the elevated serum hexosamine levels (Boas and Soffer, 1951). On theoretical grounds cortisone should, therefore inhibit the development of atherosclerosis if connective tissue ground substance breakdown is important in its pathogenesis. Such an inhibition of atherogenesis by both cortisone and hydrocortisone has been demonstrated in cholesterol-fed rabbits (Wang *et al.*, 1955, and Adlersberg *et al.*, 1954). These latter workers inferred, however, that the cortisone effect was due to diminished tissue permeability. Hyaluronidase in the present experiment increased atheroma formation on the aortic valves and enhanced the formation of intimal foam cell plaques. It could well have effected these changes by accentuating the process of mesodermal ground substance breakdown. Hyaluronidase has been shown to increase atherosclerosis in hypercholesterolaemic rabbits by other workers (Benditt *et al.*, 1950; Seifter *et al.*, 1953; and Wang *et al.*, 1955). Further, it has been shown that concomitant cortisone administration inhibits the atherogenic effect of hyaluronidase (Wang *et al.*, 1955).

Marked focal and discrete increases in metachromatic staining and P.A.S. positive material such as were found in association with the lesions in this experiment have also been observed in human atherosclerosis (Taylor, 1953, and Moon and Rinehart, 1952), and in animals by Rinehart and Greenberg (1951); Lindsay *et al.* (1952a, 1952b, 1955). McMillan *et al.* (1954) were, however, unable to demonstrate such an increase at the site of lesions in cholesterol-fed rabbits. Whether or not the hypercholesterolaemia induces these changes in humans remains to be answered. Recently, Antonini and Salvini (1957) and Schwartz and Gilmore (1958) have been able to demonstrate that in human atherosclerosis there is a marked elevation of both the serum hexosamine and mucoprotein levels. These findings, when considered in conjunction with the histochemical findings, strongly suggest that mesodermal ground substance breakdown may be of significance in human atherogenesis. The finding in this present work that cholesterol itself is capable of inducing a similar change in the tissues of the rabbit is therefore of considerable interest. It may be that cholesterol feeding is merely one of many ways in which tissue damage may be induced and that this latter is of greater importance in atherogenesis.

SUMMARY.

The intramuscular administration of testicular hyaluronidase produced a significant elevation in the serum hexosamine level of hypercholesterolaemic rabbits.

Hypercholesterolaemia likewise produced a significant elevation in rabbit serum hexosamine level.

It is suggested that this latter rise was due to connective tissue ground substance breakdown induced by the hypercholesterolaemia, while the former reflected the chondroitinase action of hyaluronidase.

Increased metachromatic and P.A.S. positive materials were observed in and

subjacent to atheromatous lesions. This mucoid material was hyaluronidase resistant *in vivo*.

Increased atherogenesis on the aortic valves and an enhanced foam cell plaque formation with hyaluronidase were noted.

Cartilaginous metaplasia and areas of medio-necrosis were observed in the aortae.

The significance of the above findings has been discussed with reference to the rôle of connective tissue ground substance breakdown in the pathogenesis of atherosclerosis.

Acknowledgments. We are indebted to Professor Sir C. Stanton Hicks of the Department of Human Physiology and Pharmacology for advice and encouragement, to the Medical Research Committee of the University for the supply of hyaluronidase, and to Miss M. Redway for technical assistance.

REFERENCES.

Adlersberg, D., Schaefer, L. E., and Wang, C-I. (1954): Science, 120, p. 319.
Altshuler, C. H., and Angevine, D. M. (1951): Amer. J. Path., 27, p. 141.
Antonini, F. M., and Mininni, G. (1955): Arch. Ist. biochim. ital., 17, II.
Antonini, F. M., and Salvini, L. (1957): Bull. schweiz. Akad. Med. Wiss., 13, p. 216.
Benditt, E. P., Schiller, S., Wong, H., and Dorfman, A. (1950): Proc. Soc. exp. Biol., 75, p. 782.
Boas, N. F., and Soffer, L. J. (1951): J. clin. Endocrinol., 11, p. 39.
Brown, W. D. (1954): Austral. J. exp. Biol., 32, p. 677.
Day, A. J., and Peters, J. A. (1958): *Ibid.*, 36, p. 121.
Day, A. J., and Wilkinson, G. K. (1956): *Ibid.*, 34, p. 423.
Faber, M. (1949): Arch. Path., 48, p. 342.
Kelly, F. B., Taylor, C. B., and Hass, G. M. (1952): *Ibid.*, 53, p. 419.
Lindsay, S., Feinberg, H., Chaikoff, I. L., Entenman, C., and Reichert, F. L. (1952a): A.M.A. Arch. Path., 54, p. 573.
Lindsay, S., Chaikoff, I. L., and Gilmore, J. W. (1952b): *Ibid.*, 53, p. 281.
Lindsay, S., and Chaikoff, I. L. (1955): A.M.A. Arch. Path., 60, p. 29.
McMillan, G. C., Klatzo, I., and Duff, G. L. (1954): Lab. Invest., 3, p. 451.
Meyer, K.: Connective Tissue in Health and Disease: E. Munksgaard, ed., Copenhagen (1954), p. 54.
Moon, H. D., and Rinehart, J. E. (1952): Circulation, 6, p. 481.
Oester, Y. T., Davis, O. F., and Friedman, B. (1955): Amer. J. Path., 31, p. 717.
Prior, J. T., and Hutter, R. V. P. (1955): *Ibid.*, 31, p. 107.
Rinehart, J. F., and Greenberg, L. D. (1951): Arch. Path., 51, p. 12.
Schlamowitz, S. T., De Graff, A. C., and Schubert, M. (1950): Circulation, 1, p. 822.
Schlichter, J. G., Katz, L. N., and Meyer, J. (1949): Amer. J. med. Sci., 218, p. 603.
Schwartz, C. J., and Gilmore, H. R. (1958): Circulation. In the press.
Schwartz, C. J., and Gilmore, H. R. (1958): In preparation.
Seifter, J., Baeder, D. H., Beckfield, W. J., Sharma, G. P., and Ehrich, W. E. (1953): Proc. Soc. exp. Biol., 83, p. 468.
Taylor, H. E. (1953): Amer. J. Path., 29, p. 871.
Wang, C-I., Schaefer, L. E., and Adlersberg, D. (1955a): Endocrinol., 56, p. 628.
Wang, C-I., Schaefer, L. E., and Adlersberg, D. (1955b): Circul. Res., 3, p. 293.
West, R. W., and Clarke, D. H. (1938): J. clin. Invest., 17, p. 173.
Zlatkis, A., Zak, B., and Boyle, A. J. (1953): J. lab. clin. Med., 41, p. 486.

ATHEROSCLEROSIS AND THE SERUM MUCOPROTEIN LEVELS OF THE AUSTRALIAN ABORIGINE[1]

by COLIN J. SCHWARTZ[2] AND J. R. CASLEY-SMITH

(From the Department of Human Physiology and Pharmacology and the Department of Anatomy, University of Adelaide).

(*Accepted for publication 27th November, 1957.*)

It has been shown by Antonini and Salvini (1957) and by Schwartz and Gilmore (1958) that in human atherosclerosis there is an elevation of the serum mucoprotein and hexosamine levels. Further, that the serum levels of both fractions increase with ageing. By the *in vivo* enzymatic depolymerization of connective tissue mucopolysaccharides with hyaluronidase in rats marked elevations of both the serum mucoprotein and hexosamine levels have been demonstrated (Schwartz and Gilmore, 1958). In this way, the possible clinical significance of these fractions has been studied. Elevations of the serum mucoprotein fraction have been noted in many disease states. In mice bearing transplantable tumours (Catchpole, 1950), in scurvy (Pirani and Catchpole, 1951), and in the so-called collagen group of disease including rheumatic fever, rheumatoid arthritis and disseminated lupus erythematosis by Altshuler and Angevine (1951). A factor common to all these conditions would appear to be increased depolymerization of connective tissue ground substance mucopolysaccharides.

Elevation of the serum mucoprotein levels in atherosclerosis together with a concomitant alteration in the metachromasia of arterial mucoid substances is interpreted as reflecting an increased depolymerization of ground substance in this disease (Schwartz and Gilmore, 1958). As the primitive Australian aborigines are an ethnic group with a probable low incidence of atherosclerosis, it was considered that a study of the rate of mucopolysaccharide depolymerization as measured by the serum mucoprotein levels might be of further aid in evaluating the rôle of mesenchymal breakdown in this disorder. Furthermore, by investigating an equivalent group of aborigines who have been urbanized for some years, the effect of "civilization" might also be evaluated.

---

[1] The material for this work was collected by an Anthropological Expedition from the University of Adelaide, financed largely by the Wenner-Gren Foundation for Anthropological Research Inc., New York, and by the University of Adelaide.

[2] Medical Research Fellow, National Health and Medical Research Council of Australia.

MATERIALS AND METHODS.

Three groups of healthy normal male subjects were investigated in this study. The first group consisted of 22 full-blooded tribal male Pintubis living nomadically in the Western Desert of Central Australia. The age range for this group varied from 18 to 55 years (mean 32 years). Within the second group there were 21 full-blooded male Australian aborigines of mixed tribal origins, being mainly Pintubis, Pitjantjaras and Arandas. This latter group was detribalized for five or more years and had acquired dietary and living habits much closer to those of the white people in the area. For this group the age range was from 12 to 65 years (mean 36 years). The third group studied consisted of 38 Australian males of European descent whose ages varied from 19 to 70 years (mean 37 years). These were normal, healthy people, normotensive and without any clinical evidence of atherosclerotic disease or any other condition known to effect the serum mucoprotein levels.

Venous blood samples collected from the aborigines were refrigerated immediately and after centrifugation the sera were despatched to the laboratory by air in vacuum flasks.

Serum mucoprotein was determined using a modification of the original method of Winzler (1948). 0·2 ml. of serum was used and protein was determined by the sensitive method of Lowry *et al.* (1951). Mucoprotein was expressed as mg./100 ml. of serum using human albumin as a standard.

Dietary assessment. The diet of the tribal nomadic Pintubis of the first group is essentially a simple bush diet. As cited in a previous communication by Schwartz *et al.* (1957) this differs from the civilized diet in its paucity of animal fats. Somewhat less than 10 p.c. of the Calories of the Pintubis' diet is derived from animal fats. The diet is also deficient in carbohydrate, but is adequate in both protein and vitamin contents. The second group of aborigines living near Alice Springs subsist on essentially the same diet as white Australian controls, although their fat intake is not as great, but is substantially greater than that of the first group.

Statistical analysis. The results of this work were subjected to simple t-tests.

Atheroma incidence in the Australian aborigine. Atheroma is not found either with great frequency or severity in the Australian aborigine. In a series of forty-four autopsies little or no aortic atherosclerosis was noted (Ireland, 1957). Such a finding contrasts with the almost universal finding of extensive atherosclerosis found at autopsy in European Australians.

RESULTS.

All data are summarized in Table 1 from which it can be readily seen that

TABLE 1.

The serum mucoprotein levels of primitive and "urban" aborigines compared with those of white controls.

	Primitive, Nomadic aborigines	"Urban" aborigines	White controls
Number of subjects	22	21	38
Mean age in years	32	36	37
Serum mucoprotein (mg. per 100 mls.)	74·3	93·6	90·1
Estimated standard error of mean	2·38	4·22	2·06

the mean serum mucoprotein level for nomadic tribal Pintubis (74·3) is considerably lower than the mean level of the urban group of full-blooded detribalized aborigines (93·6). The difference between these two groups of comparable ages is highly significant ($p < 0{\cdot}001$). It can further be seen that the first group of nomadic Pintubis also have a lower mean serum mucoprotein level (74·3) than a group of normal white Australians (90·1) of comparable ages. This difference between nomadic tribal natives and white Australian controls is also highly significant ($p < 0{\cdot}001$). The small difference between detribalized aborigines (93·6) and white Australian controls (90·1) is not significant. There is little difference between the mean ages of the three groups (Table 1).

DISCUSSION.

If the low incidence in aborigines of atheroma and atherosclerotic heart disease found in the small series of autopsies collected by Ireland (1957) is representative of the general aboriginal population, this is in contrast with the high incidence of atherosclerosis in the white Australian population. It has already been shown that this ethnic group has a considerably lower serum cholesterol level than that of white Australians, and this may possibly account for the lower incidence (Schwartz *et al.*, 1957). Recently, however, it has been demonstrated by Antonini and Salvini (1957), Schwartz and Gilmore (1958), that in atherosclerosis the serum mucoprotein level is elevated above that of non-atherosclerotic controls of a comparable age group. In the findings of the latter authors this elevation in atherosclerosis is highly significant ($p < 0{\cdot}001$). An elevation of the serum mucoprotein in rats has been shown by Schwartz and Gilmore (1958) to reflect an *in vivo* depolymerization of connective tissue ground substance mucopolysaccharides. The elevated serum mucoprotein and hexosamine levels in atherosclerosis are considered to reflect a similar process, analogous to the specific enzyme substrate reaction mentioned above.

If depolymerization of mesodermal connective tissue ground substance is involved in the pathogenesis of atherosclerosis, then one might expect that in an ethnic group with a low incidence of atheroma the extent of depolymerization might be lower. That this is so is evident from the lower level of serum mucoprotein in the nomadic tribal Pintubis. Of particular interest also is the finding that detribalization of the aborigine and the acquisition of the white man's diet and living habits is associated with an elevation of the serum mucoprotein levels above those low levels of the nomadic group, to approximately the same values as the white controls (Table 1). It would be only speculation if an attempt were made to account for this increase in level in the urban group, other than to say that some difference between the nomadic tribal environment on one hand, and the urban environment on the other, possibly dietary, is involved. The fact that the urban group has a higher level of serum mucoprotein than the nomadic Pintubi group precludes the possibility of an ethnic constitutional factor to account for the difference between aborigine and white Australian.

Unfortunately, no comparisons between the two groups, nomadic Pintubi and urban aborigine, with respect to the incidence of atherosclerotic disease can be made at present. Nevertheless, the findings of this investigation do in fact support the idea that connective tissue ground substance breakdown may play a rôle in the pathogenesis of human atherosclerosis. Furthermore, it can be seen that the acquisition of "civilization" increases this process. But what factor or factors are concerned, and whether this process is causal or merely occurs *pari passu* in atherogenesis, is a fundamental question which remains to be answered.

SUMMARY.

The nomadic tribal Australian aborigine has been shown to have a highly significant depression of the serum mucoprotein level when compared with an urban civilized group of aborigines on the one hand, or a group of white Australian controls on the other.

It is unlikely that an ethnic constitutional factor can account for the difference between the mean levels in the nomadic Pintubi and white Australian controls.

A possible relationship between the low levels of the serum mucoprotein in the Pintubi group and the higher levels in the white controls is discussed in terms of the possible rôle of connective tissue ground substance derangements in human atherosclerosis.

Acknowledgments. We are indebted to Professor Sir C. Stanton Hicks for his invaluable advice and criticism; to Professor A. A. Abbie for his permission and co-operation in the collection of blood samples, and to Miss M. Redway and Mr. J. A. Peters, D.M.T., for technical assistance.

REFERENCES.

Altshuler, C. H., and Angevine, D.M. (1951): Amer. J. Path., 27, p. 141.
Antonini, F. M., and Salvini, L. (1957): Bull. schweiz. Akad. Med. Wiss., 13, p. 216.
Catchpole, H. R. (1950): Proc. Soc. exp. Biol., 75, p. 221.
Ireland, J. A. (1957): Personal communication.
Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951): J. biol. Chem., 193, p. 265.
Pirani, C. L., and Catchpole, H. R. (1951): A.M.A. Arch. Path., 51, p. 597.
Schwartz, C. J., Day, A. J., Peters, J. A., and Casley-Smith, J. R. (1957): Austral. J. exp. Biol., 35, p. 449.
Schwartz, C. J., and Gilmore, H. R. (1958): Circulation. In the press.
Schwartz, C. J., and Gilmore, H. R. (1958): In preparation.
Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M. (1948): J. clin. Invest., 27, p. 609.

OBSERVATIONS ON CLEARING FACTOR INHIBITOR ELABORATED BY CORTISONE IN RABBITS

by ALLAN J. DAY AND J. ANDREW PETERS

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

(*Accepted for publication 13th December, 1957.*)

It is well recognised that the administration of heparin to animals with alimentary lipemia is associated with a reduction in plasma turbidity (Hahn, 1943) and in plasma lipid levels (Brown, 1952; Grossman *et al.*, 1954; Day *et al.*, 1957a). This effect is related to the elaboration by heparin of lipemia clearing factor (lipoprotein lipase) (Anfinsen *et al.*, 1952; Korn, 1955a, 1955b). This enzyme brings about clearing of turbidity and changes in plasma lipid content by its primary lipolytic action on the triglycerides (Shore *et al.*, 1953; Robinson and French, 1953; Grossman *et al.*, 1954). Recently it has been shown (Day and Peters, 1958a) that in lipemia resulting from uranium acetate administration to rabbits the injection of heparin is not associated with the development of clearing factor activity, this being suppressed by the presence of a clearing factor inhibitor in the uranium treated plasma (Day and Peters, 1958b). This inhibition of clearing activity, however, is not associated with a corresponding inhibition of fatty acid changes, the total fatty acids being reduced despite the presence of the inhibitor. In order to investigate further this apparent inhibition of clearing factor without concomitant inhibition of fatty acid changes it was decided to re-examine the position using other substances known to influence heparin induced lipemia clearing. Constantinides *et al.* (1955) have demonstrated that heparin fails to clear turbidity or to reduce hypercholesterolemia in rabbits receiving intramuscular cortisone. This effect is interpreted as being due to the reduced formation of clearing factor in the presence of cortisone (Cairns and Constantinides, 1955). Moreover, the elaboration of a clearing factor inhibitor by cortisone has been suggested by Seifter and Baeder (1954) although their results can be explained by direct inhibition of clearing activity by cortisone itself.

It was decided, therefore, to investigate the inhibitory effect of cortisone on the various aspects of heparin induced lipemia clearing. Two objects were in mind, first to clarify the action of cortisone on clearing factor, whether the inhibition was due to cortisone itself or to a secondary clearing factor inhibitor produced by it. The second object was to determine whether cortisone inhibited

the lipolytic changes produced by heparin as well as the turbidity changes, i.e., whether the dissociation of lipolysis and clearing factor activity was true of cortisone hyperlipemia as it was of uranium hyperlipemia. In this paper, therefore, the effect of heparin on the free fatty acids, plasma turbidity and on the level of clearing factor was determined in rabbits pre-treated with cortisone and the results compared with normal controls.

METHODS.

Experiment 1. Eighteen mongrel rabbits of mixed sexes varying in weight from 1·33 kg. to 2·08 kg. were randomly divided into pairs. The subsequent experiment was then performed using one pair of rabbits at a time. One member of the pair received intramuscularly 5 mg. (0·2 ml.) cortisone (or hydrocortisone) acetate daily, while the control member received an equivalent volume of normal saline. At the end of 14 days' treatment each animal was anaesthetised with Nembutal and ether, the carotid artery cannulated with a polythene cannula and 10 ml. blood removed. Subsequently, 1000 units of heparin were administered intravenously and 15 minutes later a further 10-15 ml. blood taken. Both samples were taken into chilled oxalated tubes, and the plasma following centrifugation (3000 r.p.m. for 15 minutes) kept at 4° C. Lipemia clearing factor, free fatty acids and plasma turbidity were determined in each sample in the case of both control and test animals. The determinations were done as soon as practicable after the blood was taken, and in any case within 4 hours. In addition, the presence of clearing factor inhibitor in the cortisone treated animals was assessed by determining the clearing factor activity of the post-heparin control plasma when mixed with the pre-heparin control plasma and comparing it with that of the same specimen mixed with the pre-heparin cortisone treated plasma.

Two concentrations of "inhibitor" were used.

C. 0·25 ml. control post-heparin plasma + 0·25 ml. control pre-heparin plasma.
D. 0·25 ml. control post-heparin plasma + 0·25 ml. cortisone pre-heparin plasma.

F. 0·4 ml. control post-heparin plasma + 0·1 ml. control pre-heparin plasma.
G. 0·4 ml. control post-heparin plasma + 0·1 ml. cortisone pre-heparin plasma.

It was thus possible to compare the inhibition present in the cortisone treated animal with the base line level present in the control.

The experimental procedure in all nine pairs of rabbits was as described, five rabbits receiving cortisone acetate and four hydrocortisone acetate.

Experiment 2. In order to assess the possible *in vitro* inhibition of clearing factor by hydrocortisone, various concentrations of soluble hydrocortisone succinate (Solu-Cortef, Upjohn) were mixed with control post-heparin plasma containing clearing factor. Five concentrations were used, 0·4 ml. active plasma being mixed with 0·1 ml. distilled H_2O containing respectively 0, 0·1 mg., 0·25 mg., 0·5 mg. and 1·0 mg. of hydrocortisone succinate. Clearing factor activity was determined at each concentration.

Experiment 3. *In vivo* inhibition of clearing factor activity by acute administration of a single dose of hydrocortisone intravenously was determined as follows: Six rabbits (1·97 kg.-2·50 kg.) were paired, one of each pair being given the hydrocortisone and the other serving as a control. The animals were anaesthetised, the carotid artery cannulated and an initial blood sample taken. The test animal then received 20 mg. soluble hydrocortisone

and the control animal an equivalent volume of saline. Thirty minutes later a second sample was taken, 1000 units of heparin administered and a post-heparin sample obtained 15 minutes afterwards. Similar conditions with regard to collection and storage of the plasma were observed to those described for Experiment 1. Clearing factor activity was determined in pre- and post-heparin samples from both test and control animals. In order to determine whether clearing factor inhibitors were elaborated 30 minutes after administration of hydrocortisone the following procedure was adopted. Using 0·25 ml. control post-heparin plasma as a source of clearing factor the inhibitor activity of 0·25 ml. of the plasma before hydrocortisone was compared with that of 0·25 ml. plasma of same animal 30 minutes after hydrocortisone. Clearing factor activity was determined in the usual way in the two comparable specimens.

Clearing Factor Activity. Lipemia clearing factor was determined as previously described (Day and Peters, 1958a) using a volume of 0·5 ml. of plasma. This amount was either 0·5 ml. of the unmixed plasma to be tested or in the case of the inhibitor studies was made up of a mixture of active post-heparin plasma with plasma containing possible clearing factor inhibitor as set out above.

Free Fatty Acids. These were determined in the plasma shortly after its collection, the plasma being meanwhile stored at 4° C. The method used has been described by Grossman *et al.* (1955a) the only modification being that the titrations were carried out in an atmosphere of nitrogen.

Plasma Turbidity. The turbidity of both pre- and post-heparin samples was read directly in a Unicam S.P. 600 spectrophotometer at 560 mμ using 2 mm. cuvettes. The plasma was undiluted and the results were recorded as direct optical density readings.

RESULTS.

The effect of cortisone and hydrocortisone on heparin induced lipemia clearing. The effect of pre-treatment with cortisone and hydrocortisone on the clearing factor activity developed following heparin injection is shown in Fig. 1. The curves presented show the mean clearing factor activity of the nine animals used in each group. It is apparent that in the rabbits that had been treated with cortisone or hydrocortisone for 14 days there was no clearing factor activity present following administration of 1000 units of heparin. In contrast is the definite clearing activity shown in the post-heparin plasma of the control animals. Figs. 2 and 3 demonstrate that this reduction in clearing factor activity in the test group is due to the presence of clearing factor inhibitor and not merely to a lack of production of clearing factor in the presence of cortisone. Fig. 2 represents the composite curves of the nine animals in each group. 0·25 ml. of post-heparin plasma being mixed with 0·25 ml. of pre-heparin control plasma and the activity compared with that when the same volume is mixed with 0·25 ml. of pre-heparin cortisone plasma. In Fig. 3, 0·4 ml. post-heparin control plasma was mixed respectively with 0·1 ml. pre-heparin control plasma and 0·1 ml. pre-heparin cortisone treated plasma and the resulting clearing factor activity compared. Seven pairs of rabbits were used for this observation and Fig. 3 is the composite curve of these seven pairs. In both Figs. 2 and 3 actual inhibition of clearing factor activity has been produced by the cortisone treated plasma. This is perhaps most markedly shown in Fig. 3 where the larger amount of

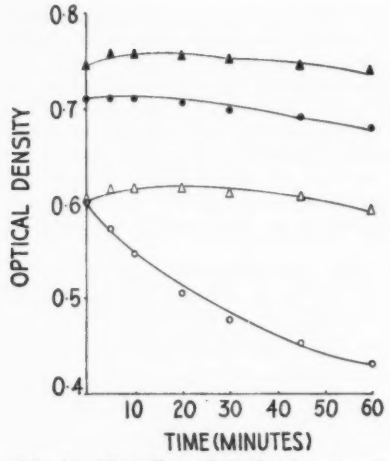

Fig. 1. The effect of 14 days' treatment with intramuscular cortisone (or hydrocortisone) on lipemia clearing activity induced by heparin in normally fed rabbits. Mean of nine rabbits in each group.
○—○ Control: post-heparin.
△—△ Control: pre-heparin.
●—● Cortisone: post-heparin.
▲—▲ Cortisone: pre-heparin.

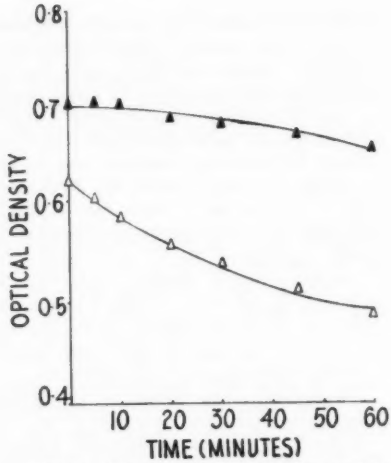

Fig. 2. Clearing factor inhibitor activity in the plasma of rabbits receiving intramuscular cortisone (or hydrocortisone) for 14 days. Mean of nine rabbits.
△—△ Control: 0·25 ml. active control post-heparin plasma + 0·25 ml. control pre-heparin plasma.
▲—▲ Cortisone: 0·25 ml. active control post-heparin plasma + 0·25 ml. cortisone treated pre-heparin plasma.

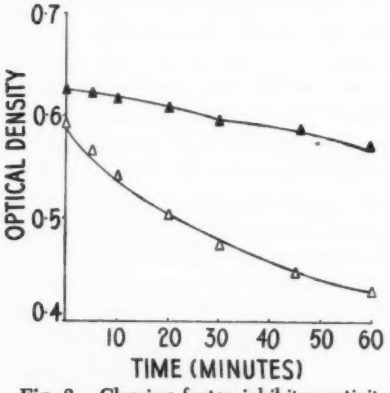

Fig. 3. Clearing factor inhibitor activity in the plasma of rabbits receiving intramuscular cortisone (or hydrocortisone) for 14 days. Mean of seven rabbits.
△—△ Control: 0·4 ml. active control post-heparin plasma and 0·1 ml. control pre-heparin plasma.
▲—▲ Cortisone: 0·4 ml. active control post-heparin plasma and 0·1 ml. cortisone pre-heparin plasma.

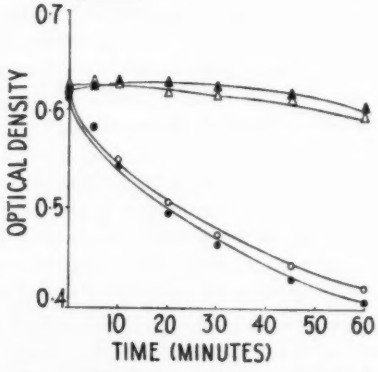

Fig. 4. The effect of intravenous hydrocortisone on the lipemia clearing activity induced by heparin 30 minutes later. Mean of three rabbits in each group.
○—○ Control: post-heparin.
△—△ Control: pre-heparin.
●—● Hydrocortisone: post-heparin.
▲—▲ Hydrocortisone: pre-heparin.

clearing factor (0·4 ml. active plasma) serves to increase the activity in the control observations, but this activity is almost completely eliminated by 0·1 ml. of cortisone treated plasma.

In order to determine whether the presence of hydrocortisone *per se* in the plasma inhibited clearing factor activity, the effect of 20 mg. intravenous hydrocortisone, administered 30 minutes before the heparin, on the subsequent clearing factor activity was determined. Fig. 4 gives the mean pre- and post-heparin curves in both the control and hydrocortisone treated animals. These curves are the composite curves of the three animals used in each group. It can be seen that there is no difference between the groups, equivalent clearing activity being produced in both groups irrespective of the presence of the hydrocortisone. Fig. 5 demonstrates that there is no clearing factor inhibitor activity in the animals receiving hydrocortisone 30 minutes before the heparin. Again the curves of activity shown are the mean of the three pairs used for this particular experiment, 0·25 ml. control post-heparin plasma being mixed respec-

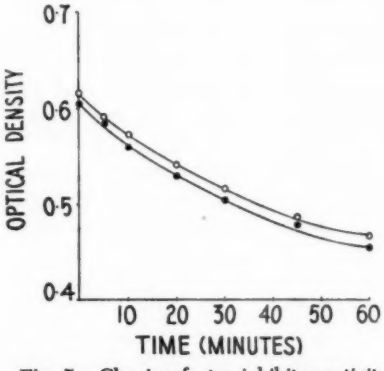

Fig. 5. Clearing factor inhibitor activity in the plasma of rabbits 30 minutes after intravenous hydrocortisone. Mean of three rabbits.
○—○ Control: 0·25 ml. active control post-heparin plasma + 0·25 ml. pre-heparin plasma before hydrocortisone.
●—● Hydrocortisone: 0·25 ml. active control post-heparin plasma + 0·25 ml. pre-heparin plasma 30 minutes after hydrocortisone.

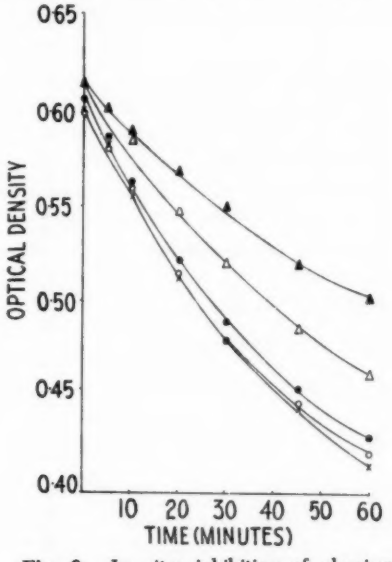

Fig. 6. *In vitro* inhibition of clearing factor activity by hydrocortisone: 0·4 ml. active control post-heparin plasma + 0·1 ml. distilled water containing 0, 0·1 mg., 0·25 mg., 0·5 mg and 1·0 mg. hydrocortisone.
×—× Control.
○—○ 0·1 mg. hydrocortisone.
●—● 0·25 mg. hydrocortisone.
△—△ 0·5 mg. hydrocortisone.
▲—▲ 1·0 mg. hydrocortisone.

TABLE 1.

Changes in free fatty acids, in vivo *turbidity and* in vitro *clearing following heparin administration in rabbits.*

Rabbit	Treatment	Sample A = Pre-heparin B = Post-heparin	Free Fatty Acids Milli-equiv./litre	Turbidity *in vivo*	Clearing *in vitro* p.c. in 30′
V 1	Control	A	0·84		—
		B	1·94		16·6
V 3	Cortisone	A	0·90		—
		B	1·22		1·2
V 2	Control	A	0·45		—
		B	0·94		14·7
V 4	Cortisone	A	0·45		—
		B	2·85		1·5
V 5	Control	A	0·18		—
		B	0·65		27·8
V 7	Cortisone	A	0·19		—
		B	1·19		0·2
V 6	Control	A	0·23		—
		B	1·34		31·9
V 8	Cortisone	A	0·57		—
		B	2·71		1·6
V14	Control	A	0·60	·042	—
		B	0·80	·031	30·9
V16	Cortisone	A	0·58	·258	—
		B	2·21	·093	5·4
X 1	Control	A	0·50	·037	—
		B	1·22	·028	18·9
X 3	Hydrocortisone	A	1·41	·400	—
		B	2·70	·290	Nil
X 2	Control	A	0·43	·035	—
		B	0·69	—	11·8
X 4	Hydrocortisone	A	1·04	·418	—
		B	3·26	·332	2·4
X 5	Control	A	0·68	·037	—
		B	0·96	·021	20·9
X 7	Hydrocortisone	A	0·87	·261	—
		B	2·25	·186	3·6
X 6	Control	A	0·63	·017	—
		B	0·79	·017	17·2
X 8	Hydrocortisone	A	0·70	·173	—
		B	1·89	·170	2·6
V11	Cortisone	A	0·52	·246	—
		B	1·70	·128	1·5
V12	Cortisone	A	0·63	·178	—
		B	1·81	·035	—
V15	Cortisone	A	0·74	·386	—
		B	1·70	·170	—
V19	Cortisone	A	0·85	·694	—
		B	1·70	·164	2·9
V20	Cortisone	A	0·87	·558	—
		B	3·54	·227	Nil

tively with 0·25 ml. pre-heparin plasma before hydrocortisone injection, or with 0·25 ml. pre-heparin plasma 30 minutes after hydrocortisone injection. The activity is similar in each group, no inhibitor activity being shown by the presence of the hydrocortisone.

The *in vitro* effects of hydrocortisone on clearing factor activity are shown in Fig. 6. The mean of 3-6 observations is presented, 0·4 ml. active plasma being used as a source of clearing factor and mixed with increasing concentrations (0, 0·1 mg., 0·25 ml. 0·5 mg. and 1·0 mg.) of hydrocortisone succinate. There is no inhibition of clearing factor activity by the lower concentrations of hydrocortisone (0·1 mg., 0·25 mg.) and only when the concentration becomes comparatively high (0·5 mg. and 1·0 mg.) is any inhibition recorded and even then is far from the complete inhibition which occurs in plasma following 14 days' treatment with hydrocortisone.

It would seem, therefore, that following hydrocortisone or cortisone administration to rabbits for 14 days there is elaborated an inhibitor to clearing factor as determined by clearing of an artificial emulsion *in vitro*. This inhibitor activity is not due to the action of the corticoid itself, but to secondary substances liberated *in vivo* during the period of treatment.

Effect of cortisone and hydrocortisone on the free fatty acid and in vivo *turbidity changes produced by heparin.* Table 1 presents the changes in free fatty acids which occurred following heparin in both the control and the cortisone treated group. These values are compared with the changes in turbidity *in vivo* which occurred in the same specimens and with the clearing factor activity of the post-heparin sample as determined by *in vitro* clearing of an artificial emulsion. The latter effect is conveniently expressed in the Table, for the sake of brevity, as the percentage clearing occurring in 30 minutes after mixing with the emulsion substrate. In addition to the nine pairs of animals described for the *in vitro* clearing studies Table 1 includes 5 further animals receiving cortisone for the purpose of additional information in comparing the three fractions measured. Several relevant observations can be made from Table 1. The increase in free fatty acid following heparin injection is much greater in the cortisone treated rabbits. Table 2 gives the mean rise in free

TABLE 2.

Mean changes in free fatty acids (together with their estimated standard error) following heparin administration in rabbits.

Group	Free Fatty Acids. Pre-heparin. (milli-equiv./litre)	Free Fatty Acids. Post-heparin. (milli-equiv./litre)	Rise in free fatty acids. (Post-Pre.) (milli-equiv./litre)
Control	0·50±0·07 (9)	1·04±0·14 (9)	0·53±0·12 (9)
Cortisone (or Hydrocortisone)	0·75±0·12 (9)	2·25±0·24 (9)	1·51±0·22 (9)

fatty acids in the two groups together with the estimated standard error. In the control group a rise of $0 \cdot 53 \pm 0 \cdot 12$ milli-equiv./litre has occurred whereas in the strictly comparable test group a rise of $1 \cdot 51 \pm 0 \cdot 22$ milli-equiv./litre has occurred. This increased effect is highly significant. The increased effect on free fatty acids in the cortisone treated group is actually related to the increased level of lipemia present in this group. If the rise in free fatty acids is plotted against the initial lipemia as expressed by the initial turbidity reading a close correlation can be seen to exist. This is illustrated by Fig. 7 where an almost linear relationship can be seen between the change in free fatty acids and the initial turbidity. Table 2 also gives the mean free fatty acid level (with its standard error) in the two groups prior to heparin and the levels after heparin. There is a non-significant difference between the 2 groups in the pre-heparin sample, but as has been outlined the rise in level following heparin and consequently the post-heparin level is very much higher in the test group.

When the free fatty acid changes in the two groups are compared with the clearing activity as determined *in vitro* on an artificial emulsion, no correlation at all exists. Fig. 8 is a scatter diagram in which the percentage clearing at 30 minutes of the post-heparin sample is plotted against the rise in free fatty acids following heparin. It can be seen that in all the cortisone treated rabbits the relatively high free fatty acid increase is associated with practically negligible

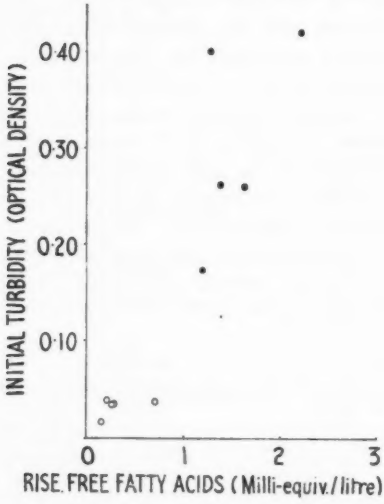

Fig. 7. Correlation of initial plasma turbidity level with the rise in free fatty acids produced by heparin in cortisone treated control rabbits.
○ Control.
● Cortisone.

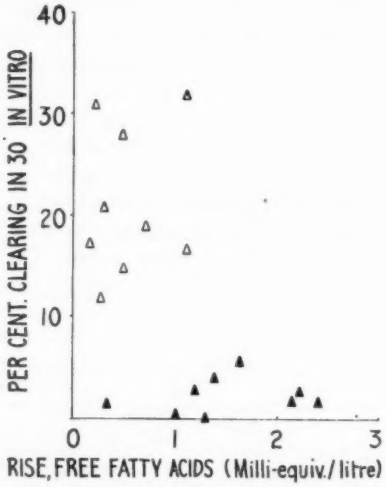

Fig. 8. Correlation of the lipemia clearing activity *in vitro* with the free fatty acid changes induced by heparin in cortisone pre-treated and control rabbits.
△ Control
▲ Cortisone.

clearing. The free fatty acid effect certainly does not give a similar picture as regards clearing factor activity to that obtained using the artificial emulsion substrate as an indicator. However, when one compares the *in vivo* turbidity reduction following heparin this is closely related to the free fatty acid increase. Thus in the cortisone treated animals, despite minimal *in vitro* clearing activity, a marked reduction in turbidity occurs when the post and pre-heparin plasmas are compared (Table 1). This *in vivo* clearing is certainly associated with the lipolytic changes as shown by the free fatty acid determinations.

DISCUSSION.

The intramuscular injection of cortisone in rabbits has been shown to produce a secondary substance capable of inhibiting the *in vitro* clearing of a fat emulsion. This substance appears following treatment with cortisone (or hydrocortisone) for 14 days, but is not present 30 minutes after the intravenous administration of hydrocortisone. Seifter and Baeder (1954) have suggested that a clearing factor inhibitor is elaborated by cortisone in rats, but their experiments do not preclude a direct inhibition by cortisone of clearing factor. The rapid appearance of inhibition in the rats they studied (1 hour after the intramuscular injection of insoluble cortisone acetate) would suggest that the cortisone itself was probably exerting the inhibiting effect. This present work, however, could exclude that possibility as hydrocortisone itself in other than very high concentration possesses no inhibitor activity. One might have expected on the basis of the work of Seifter and Baeder that intravenous soluble hydrocortisone would have produced inhibitor activity within 30 minutes, but this was not the case. The reports of Cairns and Constantinides (1955), and Constantinides *et al.* (1957), with regard to the action of cortisone on clearing activity, have been conflicting. In the earlier paper the administration of small doses of cortisone to rats prevented the development of clearing activity following subcutaneous injection of heparin. In the later work using intravenous heparin no such inhibition could be demonstrated, but only the acceleration of clearing factor activity which had been observed with high dosage cortisone. There is no really satisfactory explanation of these differing results, although it was suggested that the cortisone modified the absorption of the subcutaneous heparin in the earlier work. In any case, it would seem that in the rabbit under the circumstances of the experiment described here cortisone produced substances capable of inhibiting clearing factor activity *in vitro*. Recently it has been reported that a clearing factor inhibitor can be isolated from normal plasma (Hollett and Meng, 1957; Angervall and Hood, 1957). It might be suggested that this normally occurring inhibitor is increased in amount by cortisone.

There are several observations that can be made with regard to the free fatty acid changes. In both groups heparin has produced an appreciable elevation in the free fatty acid level. There have been conflicting reports in the past

as to whether heparin produces an effect in rabbits (Nikkilä, 1953; Schumaker, 1956; Day *et al.*, 1957b; Day *et al.*, 1957c). Certainly the direct evidence of lipolytic changes induced by heparin would strongly support the fact that this species does not differ in its response to heparin. Further, it is apparent that the lipolysis occurring as measured by the rise in free fatty acids is influenced by the level of lipemia. There is a close relationship between the initial turbidity and the subsequent increase in free fatty acids caused by heparin. Where the lipemia is high (in the cortisone group, ignoring for the moment the fact that *in vitro* inhibition occurs) there is a very much higher fatty acid response. This is in support of the findings of Grossman *et al.* (1955b) who have observed this influence of lipemia in accelerating lipolysis by heparin and of Day *et al.* (1957c), who have shown that the serum lipid response to heparin is proportional to the initial lipid level.

The apparent dissociation of clearing activity and lipolytic changes needs further comment. It has been demonstrated that cortisone inhibits the action of clearing factor in clearing an emulsion substrate *in vitro*. However, when the changes in free fatty acids occurring *in vivo* are observed no such inhibition is apparent. A similar situation to that described for uranium inhibition of clearing (Day and Peters, 1958a) has thus been shown to exist. It was previously suggested that such a dissociation of clearing and chemical changes might be due to an independent behaviour of the physical and chemical changes in the plasma, possibly due to other enzymes than clearing factor being activated by heparin (Levy and Swank, 1954, 1955). Robinson and Harris (1957), in investigating the effect of heparin on lipemia induced in the rabbit by excessive bleeding, have also shown that *in vitro* clearing may occur at a reduced rate, while lipolysis proceeds apace. However, it has been shown here that the inhibitor produced by cortisone only inhibits the *in vitro* clearing reaction. There is no indication that the reduction in *in vivo* turbidity caused by heparin has been effected by it. It would seem, therefore, that with respect to substances influencing clearing factor the substrate may be of vital importance. Thus the inhibitor described here inhibits the action of clearing factor on an artificial oil emulsion substrate but not on the plasma lipemia or on the lipolysis occurring. The problem arises as to the suitability of such artificial oil emulsion substrates for studying clearing factor activity. Although they are used quite widely it is possible that agents may influence clearing activity on this substrate but not on a more physiological one. Indeed, the normally occurring inhibitor in plasma may well be associated with only inhibition *in vitro* of an artificial oil emulsion and not an *in vivo* action. Katz (1957) has pointed out the differing effects reported when different activators and inhibitors are studied with different substrates for determining clearing activity.

The way in which inhibition of clearing of the emulsion is produced can only be speculative. Schotz *et al.* (1957) in investigating the effect of Triton on clearing factor have produced evidence that Triton inhibited clearing by

modifying the emulsion and thus preventing clearing. It is conceivable that such a physical change in emulsion properties could characterise the inhibitor activity described here. Such an effect might be specific for the oil emulsion, but not effective for lipemic plasma. It would seem therefore that care is needed in interpreting activator and inhibitor influences on clearing factor when one investigates these activities using an artificial emulsion substrate.

SUMMARY.

The effect of cortisone (or hydrocortisone) pre-treatment on the clearing activity produced by heparin injection in rabbits was recorded. The effect of heparin on the free fatty acids, plasma turbidity and the clearing factor activity determined by *in vitro* clearing was assessed under these circumstances. Both cortisone and hydrocortisone pre-treatment for 14 days prior to heparin were associated with an almost complete inhibition of clearing factor activity. This reduction was found to be due to an inhibitor substance released by the cortisone and capable of inhibiting the clearing of an oil emulsion.

Hydrocortisone *in vitro* did not inhibit clearing factor activity until very high concentrations were used. Hydrocortisone injected intravenously 30 minutes before heparin did not alter the response to heparin or liberate any inhibitor substance.

Heparin produced a rise in free fatty acids in both cortisone and control rabbits, a rise which was significantly greater in the former group and was proportional to the level of plasma lipemia. Heparin also produced a fall in plasma turbidity in both cortisone treated and control rabbits.

The dissociation of the changes produced by cortisone pre-treatment on the clearing factor activity as determined using an *in vitro* emulsion substrate and as determined by plasma turbidity and free fatty acid changes is discussed.

Acknowledgment. We are grateful to Mr. A. E. Bowey of the Pharmacy Department for preparing the emulsion substrate used and to Miss Mignon Redway for technical assistance.

REFERENCES.

Anfinsen, C. B., Boyle, E., and Brown, R. K. (1952): Science, 115, p. 583.
Angervall, G., and Hood, B. (1957): Acta. med. Scand., 157, p. 407.
Brown, W. D. (1952): Quart. J. exp. Physiol., 37, p. 75.
Cairns, A., and Constantinides, P. (1955): Canad. J. Biochem. Physiol., 33, p. 530.
Constantinides, P., Cairns, A., and So, Y. (1957): *Ibid.*, 35, p. 503.
Constantinides, P., Szasz, G., and Darrach, M. (1955): *Ibid.*, 33, p. 226.
Day, A. J., and Peters, J. A. (1958a): Austral. J. exp. Biol., in the press.
Day, A. J., and Peters, J. A. (1958b): Unpublished data.
Day, A. J., Wilkinson, G. K., Gilmore, H. R., and Schwartz, C. J. (1957a): Circulation, 16, p. 72.
Day, A. J., Wilkinson, G. K., Schwartz, C. J., and Peters, J. A. (1957b): Austral. J. exp. Biol., 35, p. 277.
Day, A. J., Schwartz, C. J., and Peters, J. A. (1957c): *Ibid.*, 35, p. 457.

Grossman, M. I., Palm, L., Becker, G. H., and Moeller, H. C. (1954): Proc. Soc. exp. Biol., 87, p. 312.
Grossman, M. I., Stadler, J., Cushing, A., and Palm, L. (1955a): *Ibid.*, 88, p. 132.
Grossman, M. I., Moeller, H. C., and Palm, L. (1955b): *Ibid.*, 90, p. 106.
Hahn, P. F. (1943): Science, 98, p. 19.
Hollett, C., and Meng, H. C. (1957): Fed. Proc., 16, p. 60.
Katz, S. (1957): J. app. Physiol., 10, p. 519.
Korn, E. D. (1955a): J. biol. Chem., 215, p. 1.
Korn, E. D. (1955b): *Ibid.*, 215, p. 15.
Levy, S. W., and Swank, R. L. (1954): J. Physiol., 123, p. 301.
Levy, S. W., and Swank, R. L. (1955): *Ibid.*, 127, p. 297.
Nikkilä, E. (1953): Scand. J. clin. lab. Invest., 5, Supp. 8.
Robinson, D. S., and French, J. E. (1953): Quart. J. exp. Physiol., 38, p. 233.
Robinson, D. S., and Harris, P. M. (1957): Biochem. J., 66, p. 18P.
Schotz, M. C., Scanu, A., and Page, I. H. (1957): Amer. J. Physiol., 188, p. 399.
Schumaker, V. N. (1956): *Ibid.*, 184, p. 35.
Seifter, J., and Baeder, D. H. (1954): Proc. Soc. exp. Biol., 86, p. 709.
Shore, B., Nichols, A. V., and Freeman, N. K. (1953): *Ibid.*, 83, p. 216.

FURTHER OBSERVATIONS ON THE SEASONAL VARIATION IN DRUG SENSITIVITY OF THE ISOLATED TOAD HEART (*BUFO MARINUS*)

by WINIFRED G. NAYLER[1]

(From the Baker Medical Research Institute, Prahran, Melbourne).

(*Accepted for publication 22nd November, 1957.*)

In an earlier study marked seasonal variation in the sensitivity of the isolated toad heart to the cardiac glycoside strophanthin-G was noted (Nayler, 1957a).

Basically the greatly diminished sensitivity associated with the period of natural hibernation could be the result of several factors such as a changed metabolic pattern of energy liberation, a lowered cell membrane permeability to the glycoside or to specific ions involved in the inotropic response, or a gross effect on the sensitivity of the contractile mechanism to the drugs or the ionic changes inherently produced by them.

In this present study some of these factors have been investigated in a further attempt to establish the mode of action of the glycosides and to determine the underlying cause of this seasonal variation in sensitivity.

In particular the effect on the inotropic response of the hibernating heart to strophanthin-G of (1) the glycogenolytic drugs dinitrophenol and sodium salicylate, (2) changes in Ca ion and K ion concentration of the perfusate, and (3) pretreatment of the heart with thyroxine, were studied. Also, since the positive inotropic response produced by 9 α-fluorohydrocortisone in the non-hibernating heart was similar to that due to the glycosides (Nayler, 1957b) the relative sensitivity of the hibernating (winter) and non-hibernating (summer) hearts to the cortisone derivative was determined.

METHOD.

Observations were made on unselected hibernating male and female toads of the species *Bufo marinus*, the toads being housed at seasonal temperatures in open cages and fresh supplies obtained at approximately monthly intervals. All experiments were carried out at $25{\cdot}0 \pm 0{\cdot}05°$ C. using the perfusion apparatus previously described for the simultaneous recording of oxygen uptake, carbon dioxide output, maximum aortic pressure, stroke output and rate of the spontaneously beating isolated heart (Nayler and McKelvie, 1956). From these observations work output and respiratory quotients were calculated at regular intervals throughout the perfusion period.

[1] The expenses of this investigation were defrayed by a grant from the Life Insurance Medical Research Fund of Australia and New Zealand.

Unless otherwise stated all preparations were perfused in standard toad Ringer during the preliminary ten-minute oxygenation and thirty-minute temperature equilibration period; observations were then made at fifteen-minute intervals during the subsequent one hour control period prior to the addition of any drugs or additional ions to the perfusate.

Metabolic Pattern.

Data from the previous experiments (Nayler, 1957a) indicated that, during natural hibernation, the respiratory quotient values of the isolated hearts (winter) fell to 0·75 from the summer level of 0·95. In view of the possibility that this altered metabolism may be partly responsible for the impaired sensitivity of the hibernating preparations to cardiac glycosides the respiratory quotients, in a series of winter hearts, were restored to 0·95 by the use of the glycogenolytic agents dinitrophenol and sodium salicylate.

In four experiments 2, 4-dinitrophenol ($1{\cdot}09 \times 10^{-5}$M final conc.) was added together with strophanthin-G[2] (1×10^{-5} final conc.) to the perfusion fluid after the initial one hour control perfusion and observations continued at fifteen-minute intervals throughout the subsequent two hours. Similarly sodium salicylate (5mM final conc.) plus strophanthin-G (1×10^{-5}) were added simultaneously to a second series of four preparations. In each case four control experiments were performed in which either dinitrophenol or sodium salicylate were added alone in the above concentrations and similar observations made throughout the initial one hour and subsequent two hours' perfusion.

Altered Drug Sensitivity.

9 α-Fluorohydrocortisone[3] (9 α FF) at low concentrations produced a positive inotropic response in the cat papillary muscle and non-hibernating isolated toad heart which was apparently identical with that elicited by the cardiac glycosides (Tanz *et al.*, 1956; Nayler, 1957b). A series of experiments was carried out to establish whether or not the isolated hibernating heart showed reduced sensitivity to 9 α FF as was the case with strophanthin-G.

In four experiments 9 α FF (1×10^{-6} final conc.) was added to the perfusate after the initial hour's perfusion; observations were continued at fifteen-minute intervals during the following two hours.

In a second series of four experiments 9 α FF (1×10^{-6}) plus strophanthin-G (1×10^{-5}) were added simultaneously to the perfusion fluid and observations recorded as above. The results were compared with those previously reported following the addition of strophanthin-G alone at this concentration to the hibernating heart (Nayler, 1957a).

Thyroxine.

Since it is generally thought that thyroxine or its metabolites play some physiological role in the hibernation pattern, a series of hearts were pre-treated with thyroxine in an attempt to restore the inotropic response.

In four preparations hearts were perfused for eighteen hours with normal Ringer to which pure L-sodium thyroxine (1×10^{-6}M final conc.) and penicillin had been initially added. After eighteen hours' perfusion these hearts were placed in the perfusion apparatus, and, following the standard one hour's control perfusion strophanthin-G (1×10^{-5}) was added as above and observations continued throughout the ensuing two hours.

Ions.

Calcium. A series of experiments was carried out to investigate the sensitivity of the hibernating heart to excess calcium ions. Four preparations using winter hearts were made hypodynamic by perfusion in half normal calcium Ringer, and following the initial one hour's perfusion, excess calcium ions (as calcium chloride) were added to the perfusate to give a final concentration of twice normal. Observations of oxygen uptake, carbon dioxide output,

---

[2] Strophanthin-G as Ouabain Arnaud: Laboratoire Nativelle Product.

[3] 9 α-Fluorohydrocortisone: Merck & Co. product.

stroke rate, maximum aortic pressure and cardiac output as indicated by number of drops per beat, were continued throughout the subsequent two hours. These results were compared with those obtained from a similar series using summer non-hibernating hearts perfused initially in half-calcium Ringer. In both instances a control series of observations were made throughout three hours perfusion in half-calcium Ringer.

In an additional series, each of four preparations, the response of the hibernating heart to strophanthin-G (1×10^{-5} final conc.) was recorded using modified Ringer containing twice normal (2·6mM/litre) and four times normal (5·2mM/litre) calcium concentration. Observations were made as above during the initial hour's perfusion and two hours following the addition of the glycoside.

Control series using the modified Ringer throughout the entire perfusion period were carried out as usual.

These results were compared with those obtained following the addition of strophanthin-G to hibernating hearts perfused with normal calcium Ringer (1·3mM/litre).

Potassium. Many investigations have indicated that the changed rate of loss of potassium ions from the myocardial cells is involved in the positive inotropic response. Since lowered external potassium concentration will facilitate the efflux of these ions, a series of four preparations were made in which strophanthin-G was added at the above concentration to winter hearts perfused in half normal potassium Ringer (1·6mM/litre) – the correct ionic equilibrium was maintained with sodium ions.

RESULTS.

In the control hibernating series using the standard perfusion fluid throughout, a relatively stable preparation was obtained which showed a gradual decline in cardiac output, maximum aortic pressure, beat rate and work output. The rate of oxygen utilization increased throughout the perfusion period and was associated with a constant respiratory quotient of 0·75.

The results obtained by the addition of the glycogenolytic agents to the perfusate differed from one another. At the concentration used both dinitrophenol and salicylate produced an increase in the respiratory quotient values from the normal hibernating level of 0·75 to 0·95, the maximum increase being apparent one hour after the addition of the glycogenolytic agent (Fig. 1A).

In Fig. 1B it is apparent that both dinitrophenol and sodium salicylate stimulated oxidative metabolism, the greatest increase occurring within forty-five minutes following the addition of either drug to the perfusate.

Whereas DNP caused no significant change in maximum aortic pressure, cardiac output, beat rate or work output sodium salicylate elicited a cardiac response similar to that previously recorded in the non-hibernating preparations (Nayler, 1957c). This response was manifested by increased maximum aortic pressure and beat output and an initial rise followed by a decline in beat rate (Fig. 1C-E). When these data were correlated to determine the change in total work output of the heart it was apparent that, although DNP restored the respiratory quotient value of the winter heart to the summer level, it did not cause any increase in the useful work output (Fig. 1F). Salicylate, however, caused a marked and sustained increase in the work done.

In Fig. 1B-F where the results obtained following the addition of strophanthin-G together with the glycogenolytic agents are displayed, it is demonstrated that the positive inotropic response has not been restored by these agents despite the raised respiratory quotients. Although strophanthin-G in the presence of salicylate caused an increase in stroke output which exceeded that produced by salicylate alone, the concomitant fall in beat rate (Fig. 1E) resulted in the changed work output level being identical with that of the salicylate control.

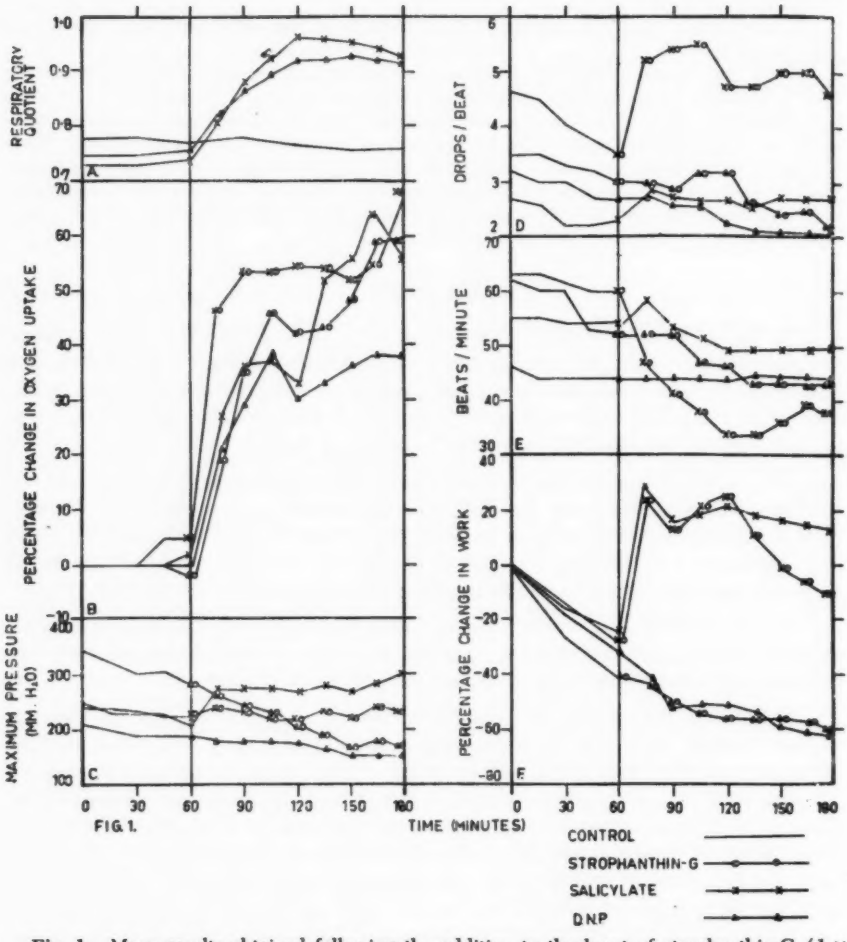

Fig. 1. Mean results obtained following the addition to the heart of strophanthin-G (1×10^{-5} final conc.) in the presence of DNP ($1{\cdot}09 \times 10^{-5}$M) — four experiments — or sodium salicylate (5mM) — four experiments. All hearts were initially perfused with standard perfusate.

Altered Drug Sensitivity. It was previously shown that 9 α FF (final conc. 1×10^{-6}) produced a positive inotropic response in the non-hibernating isolated heart preparation (Nayler, 1957b). The results obtained in this present series (Fig. 2A, F) indicate that the hibernating heart has become insensitive to this dose of 9 α FF in much the same manner as it has become insensitive to the glycosides. This reduced sensitivity has occurred despite the glycogenolytic action of, and augmented oxidative metabolism produced by, 9 α FF (Fig. 2A, F). No change in maximum aortic pressure nor increase in work output was recorded following the addition of the drug (Fig. 2D, E).

However, when both the glycoside and 9 α FF were added together at the above concentrations a well sustained positive inotropic response which resulted in an augmented work output was noted (Fig. 2E). In Fig. 2F it is evident that the pattern of oxidative metabolism displayed by the combination of these two drugs confirmed the phasic pattern previously associated with the positive inotropic response (Nayler, 1956).

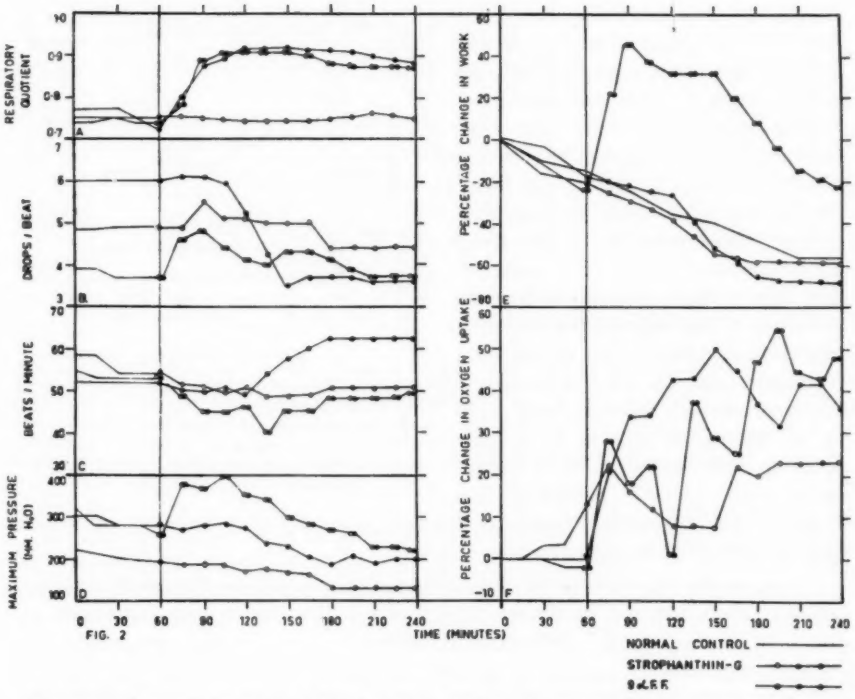

FIG. 2

Fig. 2. Observations made following the addition of strophanthin-G (1×10^{-5}) and 9 α FF (1×10^{-6}) both separately and simultaneously to hearts previously perfused with standard Ringer. In each case the mean results of four experiments are displayed.

Thyroxine. The results obtained in the thyroxine series are displayed in Fig. 3A-E. At this concentration thyroxine failed to restore the normal positive inotropic response to the glycoside, no increase in work output, maximum aortic pressure or drops per beat being recorded following the addition of strophanthin to the thyroxine treated hearts.

Calcium Ions. The addition of calcium ions to the hypodynamic hibernating heart resulted in a marked positive inotropic response associated with increased oxidative metabolism and work output. In Fig. 4A the change in work output recorded following the addition of excess calcium ions to the hypodynamic heart has been compared with that noted following the addition of the same concentration of calcium ions to the non-hibernating hypodynamic heart. Apparently hibernation markedly increased the sensitivity of the heart to these ions, and this fact was further shown when the maximum aortic pressures of the hibernating and non-hibernating hypodynamic hearts were compared. In Fig. 4B the maximum aortic pressures recorded from individual winter and summer hearts perfused in half normal calcium Ringer have been plotted, revealing the tendency for hibernating hearts to become more severely hypodynamic than their non-hibernating counterparts.

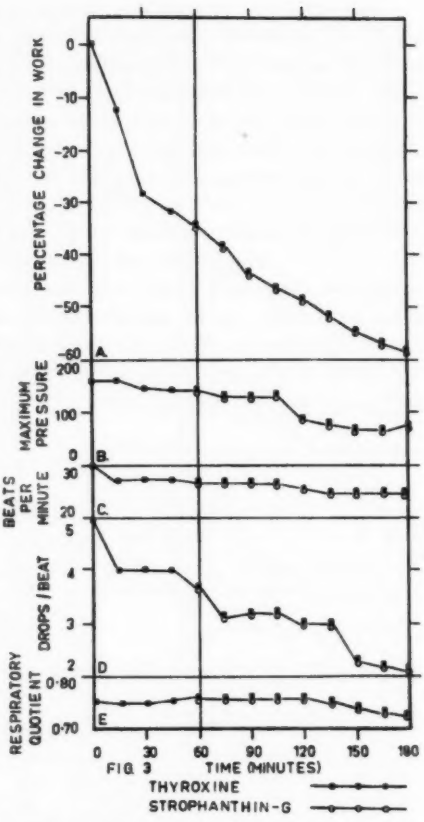

FIG. 3

Fig. 3. Mean results of four experiments in which strophanthin-G (1×10^{-5}) was added to hearts previously perfused for eighteen hours with Ringer containing L-sodium thyroxine (1×10^{-6}M) and penicillin. Note the inotropic response was not restored.

The results obtained from those preparations in which strophanthin-G was added to the hearts perfused with Ringer of varying calcium concentrations are displayed in Fig. 5A-E. Whereas no inotropic response was recorded using calcium concentrations of 1·3mM/litre or 5·2mM/litre, a well differentiated inotropic response was recorded in those preparations using 2·6mM/litre calcium concentration.

It was interesting to note that the phasic pattern of oxygen uptake was again associated with the positive inotropic response, and that the maximum percentage increase in work output recorded with this response was the same as that recorded following the administration of the same concentration of strophanthin-G (1×10^{-5}) to the non-hibernating heart perfused in standard perfusion fluid, i.e. 45 p.c. increase in work output.

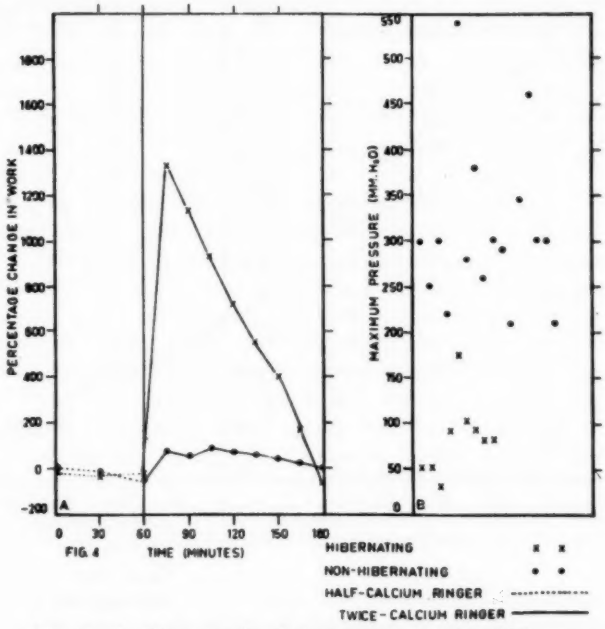

FIG. 4

Fig. 4. A. Mean results of four experiments in which excess calcium ions were added to both hibernating and non-hibernating hearts initially perfused in half calcium Ringer (0·65 mM/litre). In both series the final calcium concentration was 2·6 mM/litre.

B. Maximum aortic pressures recorded in a series of hibernating and non-hibernating hearts perfused with half normal calcium Ringer (0·65 mM/litre).

Potassium Ions. In four experiments in which the potassium concentration of the perfusate was reduced from 3·2mM/litre to 1·6mM/litre, strophanthin-G (1×10^{-5}) failed to elicit a positive inotropic response.

DISCUSSION.

In general the results reported here confirm the claim that, during natural hibernation, the sensitivity of the isolated heart to strophanthin-G is greatly reduced. A similar conclusion has now been reached regarding 9 α FF, a com-

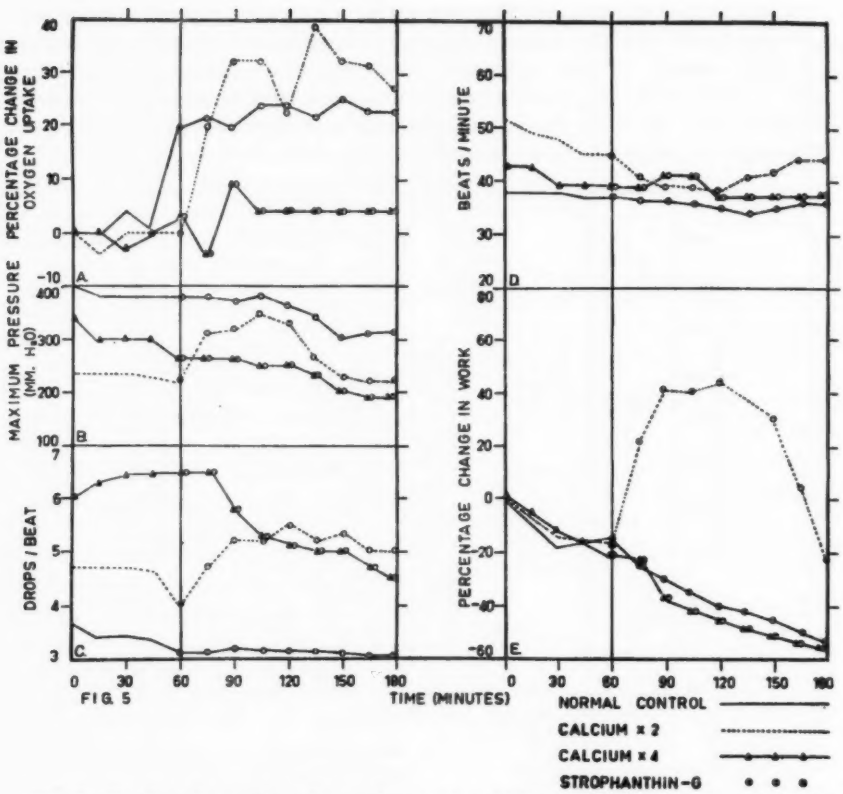

Fig. 5. Results of a series of experiments in which strophanthin-G (1×10^{-5} final conc.) was added to hearts perfused with Ringer containing normal (1·3 mM/litre), twice normal (2·6 mM/litre) and four times normal (5·2 mM/litre) calcium. Note that the inotropic response was only apparent at calcium level of 2·6 mM/litre. Mean results of four experiments are shown at each calcium concentration.

pound which in the non-hibernating heart produced an inotropic response apparently identical with that of the glycosides strophanthin-G, digoxin and lanatoside C (Nayler, 1957b). Throughout this present investigation the concentration of the inotropic drugs used has been that which, when applied to the non-hibernating heart, always produced a sustained positive inotropic response.

The finding that, when the glycoside and 9 α FF were added together to the hibernating heart, a normal inotropic response was recorded whereas individually they were inactive might suggest that the glycogenolytic activity of 9 α FF had resulted in a suitable medium in which the glycoside could produce its normal response. Alternatively the increased sensitivity could be associated

with the ionic disturbances produced by 9 α FF, since Tanz *et al.* (1956) have demonstrated that this drug increased the rate of uptake of sodium and efflux of potassium from the myocardium. It is quite conceivable that the loss of potassium ions from the cell would alter the stability of the actin-myosin complex (Szent Gyorgyi, 1952) in such a way that the contractile mechanism was rendered more sensitive to the glycoside. Newbold and Dyson (1957) have suggested that localised intracellular changes in ionic strength do modify the ATPase action, thereby influencing the relaxation/contraction cycle.

Since the other glycogenolytic agents used in this study failed to restore the response it seems unlikely that the glycogenolytic action of the cortisone derivative is the main factor in restoring the inotropic response; rather it appears more probable that the changed ionic pattern elicited by 9 α FF (Tanz *et al.*, 1956) is the principal change involved.

It is well established that DNP and salicylate (high conc.) both possess glycogenolytic activity and stimulate oxidative metabolism (Smith and Jeffrey, 1956; Brody, 1956; Sproull, 1954; Fishgold, Field and Hall, 1951; Magne, Mayer and Plantifol, 1933) and the results reported here confirm these observations.

The failure of the glycogenolytic agents to restore the normal response of the hearts to the glycoside in the series suggests that the changed metabolic pattern associated with hibernation is probably not the main cause of the reduced sensitivity. This is in agreement with the conclusion reached in a previous series in which anterior pituitary extract, when injected intramuscularly into the hibernating toad, restored the response to the glycoside without raising the respiratory quotient value to the summer level (Nayler, 1957a). The specific action of salicylate on the isolated heart is apparently similar to that previously observed following its addition at the same concentration to the non-hibernating heart.

Although thyroxine failed to restore the inotropic action in this series it is possible that one of the metabolites of thyroxine — triodothyronine — may have restored the response.

Many investigations have indicated that the cardiac glycosides increase the rate of loss of potassium from cells (Cattell and Goodell, 1937; Wedd, 1939; Regan, Talmers and Hellems, 1956; Solomon, Gill and Gold, 1956; Joyce and Weatherall, 1955). Glynn (1956) using radioactive tracers demonstrated that the passage of potassium ions across the cell membrane consisted of a small passive component proportional to the external concentration and a larger active component dependent upon some mechanism which becomes saturated at high external potassium concentrations. Since reduced external potassium would aid the efflux of the ions from the cell and make their re-entry more difficult, it might have been expected that the reduced external potassium concentration used in part of this series would facilitate the inotropic activity of strophanthin-G, provided that the increased rate of loss of potassium from the cardiac cells noted by the above workers was intimately connected with the

inotropic response. Recently, Harris and Hutton (1956) have demonstrated that the movement of potassium ions across the cell membranes was greatly increased by acetyl choline and Mutinoyi Kowo (1956) has shown increased cardiac choline esterase activity during hibernation in the frog.

From the results of these last two papers the possibility exists that the myocardial cells from the hibernating toad have decreased permeability to potassium ions as a result of the increased choline esterase activity and that the reduced permeability is associated with the lower sensitivity to strophanthin-G and 9 α FF. Seasonal variation in potassium sensitivity has been reported by other workers (Solandt, 1936; Gerard and Jennerick, 1953). However, the results obtained in this study indicate that any change in permeability to potassium ions which may be associated with hibernation is not the sole cause of the decreased response to the glycoside, since perfusion in low potassium Ringer failed to restore the positive inotropic response.

The observations made here relating to the relative sensitivity of hibernating hearts to calcium ions, and the dependence of the inotropic response upon a critical level of calcium in the perfusion fluid provide the basis for an hypothesis to partially explain the seasonal variation in drug sensitivity.

Recently Read and Kelsey (1957) have indicated that action within the myokinase system could allow digoxin to enhance the contraction at the expense of relaxation either by activating the myokinase conversion of adenosine diphosphate into adenosine triphosphate or by suppressing the myokinase inhibition of myosin ATPase activity. During normal muscular activity activation involves the exchange of localized Mg ions with Ca ions which suspends the activity of the "Marsh" factor so that ATPase activity and contraction occur. After repolarization of the membrane calcium becomes bound and magnesium free so that ATPase activity of myosin is depressed (Bozler, 1952). That Ca ions and Mg ions are bound to the contractile elements has been demonstrated by Bozler (1955), so that these ions must be considered as an integral part of the contractile mechanism. Huxley (1956) has stressed the importance of ATPase activity in any consideration of muscle contraction, the ATPase activity being increased during contraction and reduced during relaxation.

Since ATPase activity of myosin is governed in part at least by the "Marsh" factor (Weber and Portzehl, 1954) the importance of a critical calcium/magnesium ratio is evident. In view of the observation that the hibernating hearts became severely hypodynamic when placed in low calcium perfusion fluid, and since the force of contraction is proportional to the ATP concentration (Blum, Kerwin and Bowen, 1957) it can be postulated either that during hibernation the contractile system shows increased sensitivity to changes in calcium concentration or that the calcium/magnesium ratio bound to the protein has been altered in some way.

Since a positive inotropic response could only be obtained with a calcium concentration of 2·6mM/litre and not 1·3 or 5·2mM/litre it seems apparent that

there is an optimum calcium level for the contractile system above or below which the glycoside cannot produce an inotropic response. This would be so if the glycoside acted by raising the calcium level to an optimum value; such a condition would be in agreement with the conclusion reached by Read and Kelsey (1957) that digoxin had an activating effect only in a system in which myokinase activity was a limiting factor. Hence the glycosides may possibly act by raising the bound calcium to an optimum level, thereby depressing the "Marsh" factor and allowing ATPase activity to progress maximally. Niedergerke (1956) has suggested that calcium may act as "the link between depolarization and the contractile elements", and Wiercinsky (1952) found that the micro injection of calcium into isolated muscle fibres caused strong contraction, whereas magnesium caused slight relaxation indicating that the calcium/magnesium ratio governed the rate of energy release.

SUMMARY.

During hibernation the isolated spontaneously beating heart showed greatly reduced sensitivity to strophanthin-G and 9 α-fluorohydrocortisone.

Glycogenolytic agents, pre-treatment with thyroxine and reduced external potassium concentration failed to restore the response to the glycoside.

When the calcium content of the perfusate was adjusted to an optimum level a normal inotropic response was recorded.

During hibernation isolated hearts showed a marked increased sensitivity towards calcium ions.

Acknowledgment. Grateful acknowledgment is made to Dr. T. E. Lowe for his help with this study.

REFERENCES.

Blum, J. J., Kerwin, T. D., and Bowen, W. J. (1957): Archiv. Biochem. Biophys., 66, p. 100.
Bozler, E. (1952): Amer. J. Physiol., 168, p. 760.
Bozler, E. (1955): J. Gen. Physiol., 38, p. 735.
Brody, T. M. (1956): J. Pharm. exp. Ther., 117, p. 39.
Cattell, M., and Goodell, H. (1937): Science, 86, p. 106.
Fishgold, J. T., Field, J., and Hall, V. E. (1951): Amer. J. Physiol., 164, p. 727.
Gerard, R. W., and Jennerick, H. P. (1953): J. Cell. Comp. Phys., 42, p. 79.
Glynn, J. M. (1956): J. Physiol., 134, p. 278.
Harris, E. J., and Hutton, O. F. (1956): J. Physiol., 133, p. 58.
Huxley, H. E. (1956): Endeavour, 15, p. 177.
Joyce, C. R. R., and Weatherall, M. (1955): J. Physiol., 127, p. 33.
Magne, H., Mayer, A., and Plantifol, L. (1933): Amer. J. Physiol., 8, p. 1.
Mutinoyi Kowo (1956): Hirosaki Med. J., 7, p. 158.
Nayler, W. G., and McKelvie, D. (1956): Austral. J. exp. Biol., 34, p. 239.
Nayler, W. G. (1956): *Ibid.*, 34, p. 377.
Nayler, W. G. (1957a): *Ibid.*, 35, p. 131.
Nayler, W. G. (1957b): *Ibid.*, 35, p. 241.
Nayler, W. G. (1957c): *Ibid.*, in print.

Newbold, R. P., and Dyson, R. (1957): J. Physiol., 135, p. 30.
Niedergerke, R. (1956): *Ibid.*, 134, p. 584.
Read, W. O., and Kelsey, F. E. (1957): Science, 125, p. 120.
Regan, T. J., Talmers, F. N., and Hellems, H. K. (1956): J. clin. Invest., 35, p. 1220.
Smith, M. J. H., and Jeffrey, S. W. (1956): Biochem. J., 63, p. 524.
Solandt, D. Y. (1936): J. Physiol., 86, p. 162.
Solomon, A. K., Gill, T. J., and Gold, G. L. (1956): J. gen. Physiol., 40, p. 327.
Sproull, D. H. (1954): Brit. J. Pharmacol., 9, p. 121.
Szent, Gyorgyi A. G. (1952): Bull. N.Y. Acad. Med., 28, p. 3.
Tanz, R. D., Clark, G. M., and Whitehead, R. W. (1956): Proc. Soc. exp. Biol., 92, p. 167.
Weber, H. H., and Portzehl, H. (1954): Prog. in Biophysics and biophysical Chemistry, 4, p. 60.
Wedd, A. M. (1939): J. Pharm. exp. Ther., 65, p. 268.
Wiercinsky, F. J. (1952): Fed. Proc., 11, p. 172.

THE INFECTION AND GROWTH OF ECTROMELIA VIRUS IN THE CHORIO-ALLANTOIC MEMBRANE OF THE DEVELOPING HEN'S EGG

by J. J. LAWRENCE

(From the School of Public Health and Tropical Medicine, University of Sydney).

(*Accepted for publication 13th December, 1957.*)

Although the growth cycle of influenza virus in the developing egg has been studied very intensively in recent years (cf. review by Henle, 1953), there has been much less attention paid to other viruses. Of those that multiply on the ectodermal surface of the chorio-allantoic membrane detailed studies have been reported only on three: the viruses of vaccinia, lymphocytic choriomeningitis, and herpes simplex. At least four stages in the multiplication of the animal viruses can be recognised: a stage of absorption and penetration by the virus; a stage when infectivity declines (the eclipse, latent or dark phase); a stage when infectivity increases (in a step-wise fashion according to some observers) and a stage when infectivity declines. Of these stages the eclipse phase is probably the most interesting since it offers the most direct evidence that animal viruses behave like bacterial viruses in breaking down to a simpler "non-infectious" form preparatory to replicating within the cell. In fact, Burnet (1955) considers that the existence of an eclipse phase might well be the best criterion on which to define a virus. On the other hand, Bedson and Gostling (1954) and Gostling and Bedson (1956) have recently thrown doubt on the assumption that animal viruses, like bacteriophage, multiply by replication with subsequent re-assembly of non-infective sub-units. They worked with psittacosis virus, which can be perhaps excluded from the true viruses, but obtained what they regard as confirmatory results with the virus of herpes simplex. Under these circumstances it seemed worthwhile to extend such studies of multiplication to other viruses. Ectromelia virus offers some advantages in this respect since it can be titrated with reasonable accuracy in both eggs and mice (Fenner, 1948). The object of this work was to study the multiplication of ectromelia virus in the developing egg, paying particular attention to the unequivocal demonstration of an eclipse phase.

Materials and Methods.

Virus Strains.

Ectromelia. Two strains were used: Moscow, which has been kept highly virulent for mice by repeated passage; and Hampstead, which has undergone numerous egg passages.

Fenner (1949) has discussed the origin of both these strains. A stock suspension of each strain was made by homogenising with saline a number of heavily infected chorio-allantoic membranes which had been inoculated with an infected mouse liver suspension in the case of the Moscow strain and with infected chorio-allantoic membrane suspension in the case of the Hampstead strain. The homogenate was centrifuged, 50 p.c. neutral glycerol was added to the supernatant, and the stock was then kept at $-20°$ C., at which temperature it maintained its titre unchanged for many months.

Vaccinia. Strain Gillard. Received from the Walter and Eliza Hall Institute. This strain is derived from a commercial vaccinial lymph and has since been frequently passaged in rabbits.

Influenza virus type A. Strain WSE. Received from the Walter and Eliza Hall Institute. This is a variant of WS adapted to produce pocks on the chorio-allantoic membrane by Burnet and Lush (1938).

Virus Titrations.

Virus was recovered from infected tissue by homogenising in a mortar prechilled to $-20°$ C., a procedure that seems to allow maximal recovery of infective virus (Lawrence, 1957). Titrations in eggs were made by the pock counting technique as described by Beveridge and Burnet (1946). Tenfold dilutions were made in 10 p.c. inactivated horse serum saline and inoculated onto at least four eggs which were incubated for 3 days at 35° C. The results are expressed as infective units (i.u.) per ml., one i.u. corresponding to one pock. For titrations in mice an aliquot of each dilution was inoculated intraperitoneally into groups of three or four mice. Any mice surviving were killed after 13 or 14 days and their sera examined for antihaemagglutinins by the method of Fenner (1947). The titre of the virus suspension was then calculated in terms of mouse ID_{50}s by the method of Reed and Muench. To convert mouse titre to egg titre the result was divided by 10 (Fenner, 1948).

The index of dispersion of 125 sets of counts, each set consisting of 4 to 6 replicates, was calculated and showed that the counts did not conform to a Poisson distribution; indeed the variance was $12{\cdot}1 \times$ the mean. Hence, if the mean count of 5 replicates is about 50, two counts must differ by about 30 if the $p = 0{\cdot}05$ level of significance is to be reached. The error of the titrations in mice is of the same order. Using Pizzi's formula (1950), the standard error of the ID_{50} calculated by the Reed-Muench method was about 0·5 log units.

Haemagglutination test. This was based on the procedure described by Chu (1948), using 0·5 p.c. fowl cells. Cells and haemagglutinin were allowed to react for 10 minutes at 35° C., shaken and re-incubated for 30 minutes before reading. The diluent consisted of 5 p.c. inactivated rabbit serum in saline – the addition of normal rabbit serum minimizes false positive reactions.

Complement fixation test. The technique adopted was that of Donnelley (1951).

Antisera. Antiserum prepared in rabbits was preferred to mouse convalescent serum since infected tissue suspensions gave a higher titre. Antiserum against ectromelia virus was prepared by the intravenous injection of a washed deposit obtained by centrifuging a homogenate of infected mouse liver for 180,000g minutes in an angle centrifuge in the cold. That against vaccinia virus was prepared on the lines laid down by Craigie and Wishart (1934).

Receptor destroying enzyme (RDE). The method of preparation and titration followed Ada and French (1950) and of concentration, Isaacs and Bozzo (1951). Its final titre was 1/6,000.

Growth curve of virus. Eggs were each inoculated with 0·5 ml. containing the desired amount of virus, incubated at 35° C. and harvested in batches of 4-6. The dropped area of the chorio-allantoic membrane was cut out, quickly washed in saline, and homogenised by the prechilled mortar method. The homogenate was transferred together with saline washings of the mortar to a graduated centrifuge tube, the volume noted

so that subsequent virus titrations could be converted to i.u. per egg, and then centrifuged at 2,000 r.p.m. for 10 minutes. The supernatant was stored at -15 to $-20°$ C. until the titrations could be carried out. The amount of unabsorbed virus was determined for the shorter periods of incubation (up to 14 hours) by repeatedly washing the exposed dropped area of the chorio-allantois with a measured volume of serum-saline and titrating the pooled washings. The chorio-allantoic membranes were then harvested as above.

EXPERIMENTAL.

Initial attachment of virus to the chorionic cell. In the influenza-mumps group of viruses the infective particle can attach itself to a mucoprotein receptor which forms part of the surface of the susceptible cell. This specific attachment mechanism can be destroyed by the receptor destroying enzyme (RDE) of *Vibrio cholerae* (Stone, 1948). The rather remote possibility that ectromelia virus might make use of the same receptors can be checked by treating the cells of the chorio-allantoic membrane with RDE before inoculating the virus. The RDE was diluted with saline so as to contain 600 units per 0·05 ml. and this volume of the diluted RDE was inoculated onto the dropped chorio-allantoic membranes of six 11-day eggs which were then incubated for 2 hours at 35° C. Control eggs were incubated similarly after the inoculation of 0·05 ml. saline. In order to test whether the RDE had effectively destroyed the receptors a second series of eggs was inoculated with the strain WSE of influenza virus which produces pocks when inoculated onto the chorio-allantois. The virus inoculum aimed at was 25-100 i.u. contained in 0·05 ml. The results are set out in Table 1 from which it can be seen that the RDE treatment did not reduce the number of pocks produced by ectromelia virus, but that it did bring about a big reduction in the pock count of the WSE strain of influenza virus. It may be concluded that the specific mucoprotein receptors destroyed by treatment with RDE play no part in the absorption and infection of the chorionic cells by ectromelia virus.

The rate at which a virus becomes attached to the chorio-allantoic membrane may be followed by measuring the amount of virus that is removed by washing the membrane at various periods of time after inoculation. Two techniques were used. The first, which is due to Wildy (1954), has the great advantage that variations in the susceptibility of individual eggs are cancelled out since two counts are made on each egg; one gives the amount of virus immobilized and the other the amount of virus washed away. The inoculum, contained in a volume of 0·05 ml., was incubated on the dropped chorio-allantoic membrane for the desired length of time whereupon 0·1 ml. of washing fluid (10 p.c. horse-serum saline) was added and the egg carefully rotated through an angle of 120° so as to carry the free virus to a clean area of the chorio-allantois. The egg was then reincubated in this new position for three days and the number of pocks developing on both areas counted. The fraction of the inoculum immobilized was then calculated for each egg and the results

averaged. The second technique adopted consisted in incubating in inoculated egg as usual, then breaking away the shell over the dropped area of the chorio-allantois and adding 0·5 ml. of serum-saline which was pipetted up and down several times so as to wash the membrane well, care being taken to avoid making the membrane bleed. These washings were then titrated as usual. Wildy's technique can be used with ectromelia virus only when the inoculum is less than 200 i.u., but in this range it gave much the same results as the washing technique. The results obtained by these two methods using the Moscow strain are set out in Fig. 1, which also shows the rate of absorption of the Hampstead strain, but in the latter case the inoculum was $7{\cdot}9 \times 10^2$ i.u. so the conditions are not exactly comparable. However, in both cases it can be seen that absorption is practically complete after 4, if not 3, hours.

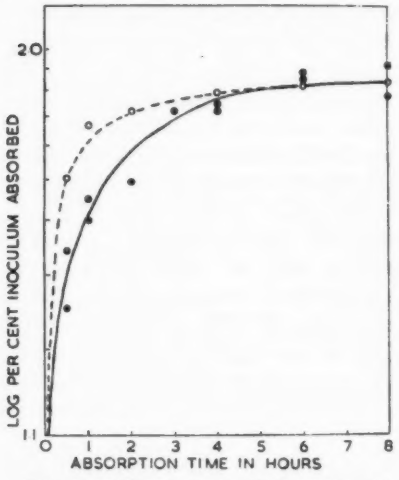

Fig. 1. The rate at which ectromelia virus is absorbed by the chorio-allantoic membrane.

○ Hampstead strain: inoculum $7{\cdot}9 \times 10^2$ i.u.

● Moscow strain
Ⴔ Moscow strain (Wildy's technique)
} inoculum 100-200 i.u.

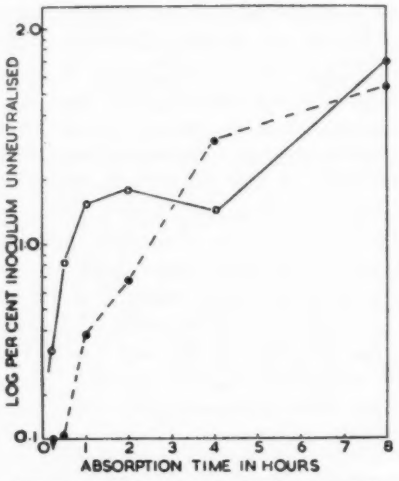

Fig. 2. The rate at which ectromelia virus becomes inaccessible to neutralising antiserum after inoculation onto the chorio-allantoic membrane.

○ Anti-ectromelia serum.
● Anti-vaccinia serum.

A number of workers have studied the rate at which a virus is absorbed by the host cell by making use of the fact that a virus particle when within the cell seems to be protected from antiserum and will continue to produce its characteristic effects as though antiserum were not present. The proportion of virus particles that have become inaccessible to the neutralising action of antiserum is measured. The method adopted was similar to that used by Burnet and Lush (1939) in their work on herpes virus. Eggs were inoculated on the

dropped chorio-allantois with about 100 i.u. contained in 0·05 ml. and, after incubation for various periods, 0·1 ml. of a rabbit antiserum was added to each of a group of six eggs. These were then returned to the incubator for three days, after which the pocks on the chorio-allantoic membrane were counted. The results of two experiments using two different antisera, one an anti-ectromelia antiserum and the other a vaccinia antiserum, are set out in Fig. 2. It may be seen that the rate of virus absorption by the cell as measured in this way is slower than that found by the use of washing techniques. Only about a quarter of the virus in the inoculum was unaffected by the addition of neutralising antiserum after 2-4 hours whereas about three-quarters could not be removed by washing at this time. Even after 8 hours 25-50 p.c. was neutralised.

The rate of growth of ectromelia virus on the chorio-allantoic membrane. Eggs were inoculated on the dropped chorio-allantoic membrane with the desired amount of virus contained in a volume of 0·05 ml. and were then incubated at 35° C. In the earlier experiments the technique for harvesting the membranes differed from that finally adopted which is described in the methods. An attempt was made to harvest any unabsorbed inoculum together with the chorio-allantoic membrane by a technique similar to that used by Tobin (1954). The hole in the shell through which the inoculum had been introduced was sealed with paraffin wax, the shell and membranes were cut around the long equator of the egg outside the area of the dropped membrane and the lower part of the shell together with the embryo was discarded. The lower (allantoic) side of the dropped chorio-allantoic membrane was then washed with sterile saline (from a wash bottle) and the membrane plus any unabsorbed inoculum was placed in a mortar. During this time the upper half of the egg had been kept in its original position, i.e. shell on top, chorio-allantoic membrane beneath. The shell was then inverted, rinsed with saline and the washings added to the mortar in the hope of picking up any residuum of the inoculum. The aim was to measure the total virus, both absorbed and unabsorbed, since virus which is removable from the earlier harvested membranes by washing may be subsequently taken up and will then contribute to the total available for multiplication. Later, say after 8 hours, it would probably be safe to wash the membranes since the great majority of the virus is absorbed by then. The results given in the first two columns of Table 2 were obtained by this technique. However, when an attempt was made to check the efficiency of this technique by harvesting the chorio-allantoic membrane immediately after inoculation the proportion of the inoculum recovered was very variable; indeed, in one experiment using a large inoculum it was as low as 18 p.c. If the virus inoculum is made up in 8 p.c. gelatin in serum-saline (which is quite fluid at room temperature but will set hard at refrigerator temperature), the membrane can be harvested without fear of losing any inoculum although some virus may be absorbed before the gelatin sets. This absorption was minimized by placing the eggs at — 20° C. immediately after inoculation. Recoveries of the inoculum were quite

good (83-135 p.c.) so it seemed that, in spite of the care taken, a variable amount of the unabsorbed inoculum was lost during harvesting when using the preceding method. For this reason the technique given in the methods was adopted in the later studies. Representative examples of these are given in the last two columns of Table 2 and in Fig. 3 (Hampstead strain).

TABLE 1.

The effect of pretreatment of the chorio-allantoic membrane with RDE on the pock counts given by the viruses of ectromelia and influenza (WSE strain).

	Pock count on chorio-allantoic membranes	
	ectromelia	influenza (WSE)
Membranes untreated	76	34
Membranes treated with RDE	73	1

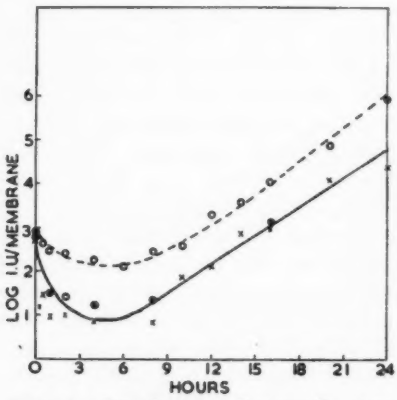

Fig. 3. Growth curve of ectromelia virus on the chorio-allantoic membrane during the first 24 hours.
○ Hampstead strain: inoculum $7{\cdot}9 \times 10^2$ i.u.
● Moscow strain: inoculum $5{\cdot}7 \times 10^2$ i.u. Tobin's technique.
× Moscow strain: inoculum $5{\cdot}4 \times 10^2$ i.u. Tobin's technique.

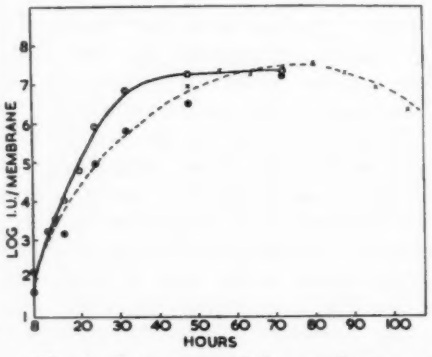

Fig. 4. Later stages of the growth curve of ectromelia virus on the chorio-allantoic membrane.
○ Hampstead strain: inoculum $7{\cdot}9 \times 10^2$ i.u.
● Moscow strain: inoculum $5{\cdot}7 \times 10^2$ i.u.
× Moscow strain: inoculum $6{\cdot}5 \times 10^2$ i.u.

The results show an eclipse period lasting for 8-10 hours followed by a period (lasting to about the twenty-fourth to thirtieth hour) during which the titre of infective virus in the membrane seems to increase in a logarithmic fashion. There is no indication of a stepwise increase in virus. The maximum titre is reached after about 48-72 hours. Both strains studied eventually kill

TABLE 2.

The growth of ectromelia virus on the chorio-allantoic membrane.

Incubation Period (hours)	Ratio of the amount of virus recovered from the chorio-allantoic membrane to the amount in the inoculum			
	Strain Moscow Inoculum $5{\cdot}4\times10^{2}$ i.u.	Strain Moscow Inoculum $3{\cdot}1\times10^{4}$ i.u.	Strain Moscow Inoculum $6{\cdot}0\times10^{2}$ i.u.	Strain Hampstead Inoculum $7{\cdot}9\times10^{2}$ i.u.
½	0·06	0·02	0·21	0·54
1	0·02	—	0·15	0·34
2	0·02	0·04	0·04	0·29
4	0·01	0·06	0·07	0·21
6	—	0·01	—	0·18
8	0·01	0·04	0·02	0·34
10	0·14	0·07	0·29	0·55
12	0·25	0·27	1·06	2·2
14	1·4	3·3	1·45	4·2
16	1·6	3·1	—	13
18	—	17·0	—	—
20	21·6	13·9	—	83
22	—	60·0	—	—
24	41·3	55·2	—	$1{\cdot}1\times10^{3}$
28	—	$1{\cdot}0\times10^{3}$	—	—
32	$2{\cdot}5\times10^{3}$	$3{\cdot}5\times10^{3}$	—	$8{\cdot}6\times10^{3}$
48	$1{\cdot}3\times10^{4}$	$1{\cdot}8\times10^{3}$	—	$2{\cdot}2\times10^{4}$
72	$3{\cdot}0\times10^{4}$	$7{\cdot}2\times10^{3}$	—	$3{\cdot}0\times10^{4}$

the chick embryo, but it proved possible to follow the Moscow strain after the inoculation of $6{\cdot}5\times10^{2}$ i.u. for 152 hours at which time all the eggs were dead. The results up to 108 hours are set out in Fig. 4 and show that the titre starts to decline after about 4 days, but at a fairly slow rate; the titre after 6 days is about 1/15 of the maximum.

Complement fixation and haemagglutination tests were carried out on the homogenates of the chorio-allantoic membrane harvested after the various periods of incubation, but the titres obtained were disappointingly low. The earliest that complement fixing antigen could be detected was at 24 hours though more usually none was found before 32 hours. From these and other results it appears that the infectivity titre of the homogenate must be about 10^{6} i.u. per ml. before a positive complement fixation test can be expected. The haemagglutinin could not be detected any earlier; in fact, it was not found until 32 hours after inoculation.

An attempt was made to demonstrate the existence of an eclipse phase by Anderson's (1954) modification of the method used by Giradi, Allen and Sigel (1952). The latter suggested that if the eclipse phase was the result of a change from infectious to non-infectious virus and back again, then infected tissue removed at intervals should have similar potentialities for producing virus even although direct titration of the infected tissue showed a decreasing amount of virus. The method employed followed Anderson closely. One 12-day egg was

inoculated on the dropped chorio-allantois with about 200 i.u. contained in a volume of 0·05 ml. and incubated for 6 hours at 35° C. The membrane was then harvested, washed quickly in Hanks' balanced salt solution and divided as accurately as possible into two halves. One half was ground by the prechilled mortar method with about 0·5 ml. of 10 p.c. horse serum saline, centrifuged at 700*g* in an angle centrifuge for 5 minutes and the whole of the supernatant inoculated in 0·05 ml. amounts onto a number of eggs. The other half was cut up into 20 small pieces of approximately the same size and each piece was placed on the dropped chorio-allantoic membrane of a 12-day egg. These were incubated as usual for 3 days when the membranes were harvested separately, homogenised and inoculated onto eggs to determine the virus content. Those homogenates that were negative or gave doubtful pocks were titrated for virus by inoculation into mice.

Two separate experiments were carried out and their results are set out in Table 3. In the first experiment the inoculum was 316 i.u., one graft failed to

TABLE 3.

Showing the relative amounts of virus liberated by homogenisation from the chorio-allantois and virus potentially capable of multiplication in the chorio-allantois six hours after inoculation.

Virus inoculated (i.u./ chorio-allantoic membrane)	Virus recovered from half the chorio-allantoic membrane	
	Infective virus in the homogenate (i.u.)	Actual plus potential infective virus (No. positive out of 20 tissue pieces)
316	0	9
180	2	15

take and 9 of the remaining 19 grafts gave counts ranging from 10^4 to 10^6 i.u. The remaining 10 grafts were negative even by mouse inoculation. The other half of the membrane which was immediately homogenised and inoculated onto eggs did not produce any pocks. In the second experiment the inoculum contained 180 i.u. and each of the pieces of infected chorio-allantois was flattened out as far as possible in a drop of Hanks' balanced salt solution placed on the surface of the dropped chorio-allantoic membrane in the hope that this would encourage the graft to take. This time twelve of the twenty grafts were positive on eggs, the titre found varying from 5 to 10^5 i.u. per membrane, and three more were positive on mouse inoculation. Hence virus was found in fifteen of twenty pieces as compared with a count of 2 i.u. given by the homogenate of the other half of the membrane.

DISCUSSION.

The rate of attachment of ectromelia virus to the chorio-allantois as measured by washing the membrane seems slower than that of those other viruses

that have been studied in this way. Wildy (1954), working with herpes virus, found that about 90 p.c. of the virus was immobilized after 1½ hours and about 95 p.c. after 4 hours. McNair Scott, Coriell, Blank and Gray (1953) considered that this virus was taken up even more quickly than Wildy's experiments would suggest: 99 p.c. of the virus was attached to the cells in the first hour. With ectromelia virus 60-70 p.c. of the inoculum was attached to the cells after 2 hours but the rate then became much slower and only 80 p.c. was absorbed after 8 hours. It may be that only about 70-80 p.c. of the virus particles in the inoculum are absorbed as appears to be the case when influenza virus is inoculated into the allantoic cavity (Henle, 1949) and that the subsequent decrease in the amount of freely movable virus is due to the progressive inactivation of the unabsorbed virus at incubator temperature. However, in the experiments using Wildy's method the total number of lesions counted on the chorio-allantoic membrane did not show any definite decrease with time and ectromelia virus suspended in the horse serum saline diluent showed no appreciable loss of titre when incubated at 35° C. for 6 hours.

The results obtained by measuring the rate of penetration of a virus into the cell by adding a neutralising antiserum have been rather variable. Wildy, working with herpes virus, found that about 35 p.c. of the virus in the inoculum remained un-neutralised after 90 minutes and that no more virus was neutralised up to 4 hours when the experiment was terminated. The only explanation that he could offer for this unexpected result was that his control count on the inoculum might have been higher than it should have been owing to the development of secondary pocks and that these did not develop in the presence of neutralising antiserum. The curve for ectromelia virus neutralised by the ectromelia antiserum (but not when the vaccinia antiserum was used) showed a plateau from 1 to 4 hours after inoculation with about 25 p.c. of the virus un-neutralised; but it then rose sharply to the 8-hour period when 75 p.c. was un-neutralised. If Wildy had continued his experiment beyond 4 hours he might have found a similar increase in the amount of un-neutralised virus. McNair Scott *et al.* also studied the rate of absorption of herpes virus in a rather complicated series of experiments in which the antiserum was added at various intervals after the eggs had been inoculated and the chorio-allantoic membrane was washed and harvested after subsequent incubation for various periods of time. Both the chorio-allantoic membrane and the washings were titrated for virus and the latter for antibody as well. They concluded from their results that the greater portion of the virus was absorbed in 10-15 minutes. On the other hand Shaffer and Enders' (1939) results indicate that herpes virus became insusceptible to the action of neutralising antiserum at a much slower rate: 4 p.c. un-neutralised after 5 minutes; 27 p.c. after 80 minutes; 24 p.c. after 3 hours; 59 p.c. after 6 hours and 100 p.c. after 18 hours. These figures are much closer to those found with ectromelia virus.

From the results given in Table 2 it will be seen that there is an eclipse or

lag phase during which there is no evidence of any multiplication of infective virus, but if anything there is a progressive diminution in the amount of virus that can be recovered. However, the errors of the titration methods are such that when the inoculum is of the order of 5×10^2 i.u. the ratios of the amount of virus recovered to the amount inoculated must vary by about 0·04 if the difference is to be significant at the $p = 0{\cdot}05$ level, so too much reliance cannot be placed on the individual figures. The duration of the eclipse phase in the growth of vaccinia virus was found by Anderson to be about 10 hours after an inoculum of 250 i.u.; but Maitland and Tobin (1956), who were not sure whether their results necessarily indicated that an eclipse phase existed, found that the virus began to increase after 4-5 hours. However, their inocula seem to have been large, ranging up to 10^7 i.u. For herpes virus, McNair Scott *et al.* found that the duration of the eclipse period varied with the size of the inoculum and was 4 hours after an inoculum of $1{\cdot}0 \times 10^8$ i.u., but was 12 hours after an inoculum of 55 i.u. Modi and Tobin (1954) and Wildy both found the eclipse phase to be about 10 hours when the inoculum was of the order of a few hundred. Hence it seems that the duration of the eclipse phase with ectromelia virus is about the same as with vaccinia and herpes viruses when they are grown on the dropped chorio-allantoic membrane.

More direct evidence for the existence of an eclipse phase is given by the results of the experiments using Anderson's modification of the technique of Giradi, Allen and Sigel. The average number of infective units expected to be present in the small pieces of grafted tissue was about five if the inoculum was uniformly distributed, so that all should have been positive. The failures are probably due to unequal distribution of virus and failure of the graft to take — it is very hard to be certain whether a graft has taken or not. In any case, much more virus was recovered by the grafting procedure than from the homogenate: at least 9 i.u. compared with none in one experiment, and at least 15 compared with 2 in the other. Thus ectromelia virus apparently goes through a phase when it cannot be liberated in an infectious form on disrupting the cells but still retains its potentialities for producing infective virus if the cells remain intact. Giradi *et al.* worked with meningo-pneumonitis virus and although their technique provides a very convincing demonstration that an eclipse phase does exist, it must be admitted that Bedson and Gostling (1954), working with the related psittacosis virus, could find no evidence of a non-infectious phase in the growth cycle. Also, Swain (1955), who studied the multiplication of the psittacosis virus in the mouse spleen and the yolk sac of the chick embryo by phase contrast and electron microscopy, concluded that this virus multiplies by binary fission. Heinmets and Golub (1948) had previously suggested on the basis of observations with the electron microscope that simple division, though not necessarily equal division, of the psittacosis virus took place. Such observations do not rule out the possibility of the existence of a non-infectious phase in the cycle of

multiplication of the psittacosis group of viruses, but they do make it harder to accept.

On comparing the phase of multiplication of the growth curves of ectromelia and vaccinia viruses some interesting points emerge. It is generally considered that ectromelia virus multiplies rather more slowly on the chorio-allantois than does vaccinia virus; for instance, with the latter virus the pocks may be easily counted after 2 days' incubation, whereas 3 days are needed before the pocks caused by ectromelia virus are countable. Even then the ectromelia pocks are somewhat smaller than the 48-hour vaccinia pocks. In conformity with this, the maximum growth rate found for ectromelia virus was about 0·2-0·25 log. infective units per hour during incubation from the twelfth to the twenty-fourth hour, while for vaccinia virus Briody and Stannard (1951) found 0·25-0·3 log. i.u. per hour (seventh to twenty-fourth hour), Anderson (1954) 0·25 log. i.u. per hour (tenth to twenty-fourth hour) and Lépine *et al.* (1951) 0·4 log. i.u. per hour (tenth to twenty-fourth hour). All the studies on vaccinia were carried out at 37° C. (or perhaps at 38-39° C. in the case of Lépine and his co-workers) as against 35° C. for ectromelia. However, Siim (1949) has made a study of the growth of vaccinia virus in the chick embryo at various temperatures and his Fig. 2 showing graphically the multiplication of the virus on the chorio-allantois indicates that there is very little difference in the slope of the curves for 34·5° C. and for 40·5° C. during the period of maximum growth.

Briody and Stannard produced evidence of a stepwise release of virus at 8 hours and at 16 hours, but Anderson found no release of virus at 8 hours, when indeed the virus was still in the eclipse phase, and considered that there was a steady liberation of virus in a logarithmic fashion from the ninth to the twenty-fourth hour. Certainly his figures do not support the idea of a stepwise increase of virus. The results obtained with ectromelia seem best interpreted by postulating a steady logarithmic production of virus from, say, the twelfth hour to the thirtieth. Any irregularities in the growth curve are well within the rather considerable errors that arise in liberating virus from tissues and in titrating on the dropped chorio-allantoic membrane. Nossal and de Burgh (1954) found that the titre of ectromelia virus showed a stepwise increase in the mouse liver after the intravenous inoculation of a massive dose of virus (10^8-10^{10} mouse LD_{50}). Thus the conditions were rather different to those studied on the egg; in particular, the inoculum was much larger. Cairns (1957) has shown that there is a highly variable delay at some stage in the infection of allantoic cells by influenza virus which is characteristic for each virus-cell encounter so that a large inoculum should tend to synchronize the release process. Perhaps a large inoculum of ectromelia virus onto the chorio-allantoic membrane would have shown production of virus in steps.

The "constant" phase and the phase of decline seem very similar to those reported for vaccinia. Lépine *et al.* (1951) found that the concentration of virus

was stationary from the 62nd to the 74th hour, after which there was a second period of growth which lasted until the death of the embryos which occurred after about 98 hours. However, Siim (1949) does not mention any such secondary rise in multiplication. When the eggs were incubated at 34·5° C. the maximum titre was reached after 41 hours and was then maintained until the embryos died after 96 hours. When the eggs were incubated at 40·5° C. the maximum was again reached after 41 hours, but the titre then slowly declined from the 60th to the 162nd hour when the titre had fallen to about half the maximum. Also, Briody and Stannard, using the titre of haemagglutinin as the criterion of growth, adopted 44 hours as the routine incubation time for harvesting since there was little or no increase in titre on leaving the eggs for 72 hours.

Hoyle (1950), working with influenza virus, found that the formation of both soluble and virus-bound complement fixing antigens preceded the rise in infectivity, and Liu and Henle (1951) found that such was also the case with the haemagglutinin. However, with ectromelia virus it appears that a considerable amount of virus multiplication must take place before these antigens can be detected. This is in line with the results obtained with vaccinia virus by Maitland and Tobin (1956), by Metcalf (1955) and by Oya (1955), and with the complement fixing antigen of herpes virus by Wildy and Holden (1954). Metcalf, using a very sensitive technique for the detection of haemagglutinin, found it present as early as 9 hours after a large inoculum (10^6 i.u.), but with a decreasing amount of virus in the inoculum the lag period increased and was 20-22 hours after an inoculum of 3×10^2 i.u. A rise in infectivity preceded any rise in haemagglutinin titre. Maitland and Tobin also found that haemagglutinin and soluble complement fixing antigen were formed during the growth of the virus and that the titre was directly related to the amount of infective virus. These two antigens were detectable when the infectivity titre reached $10^{6.5}$-$10^{7.0}$ and $10^{5.5}$-$10^{6.0}$ i.u./ml. respectively. The figures for ectromelia virus are rather similar: 10^6-10^7 i.u./ml. for haemagglutinin and about 10^6 i.u./ml. for complement fixing antigen.

SUMMARY.

The infection of the chorio-allantoic membrane of eggs by ectromelia virus and the subsequent multiplication of the virus has been studied.

Ectromelia virus seems to be taken up by the chorio-allantoic membrane somewhat more slowly than other viruses since absorption took about 4 hours as measured by washing techniques and even after 8 hours some 25 p.c. of the virus was still accessible to the action of a neutralizing antiserum.

An eclipse phase lasting for about 8-10 hours was found after inocula of $5·4 \times 10^2$ to $3·1 \times 10^4$ i.u., which was followed by a phase during which the virus showed an exponential increase and which lasted for about 14-20 hours.

The maximum titre was reached after 48-72 hours and declined at a slow rate from 96 hours onwards.

Some extra evidence for the existence of an eclipse phase was provided by the results of grafting infected tissue onto the chorio-allantoic membrane.

Complement fixing antigen and haemagglutinin could not be detected until the virus had multiplied to a considerable extent.

Acknowledgment. I wish to thank Professor P. M. de Burgh for his continued interest and advice during this work.

REFERENCES.

Ada, G. L., and French, E. L. (1950): Austral. J. Sci., 13, p. 82.
Anderson, S. G. (1954): Austral. J. exp. Biol., 32, p. 633.
Bedson, S. P., and Gostling, J. V. T. (1954): Brit. J. exp. Path., 35, p. 299.
Beveridge, W. I. B., and Burnet, F. M. (1946): Spec. Rep. Ser. med. Res. Coun. (Lond.), No. 256.
Briody, B. A., and Stannard, C. (1951): J. Immunol., 67, p. 403.
Burnet, F. M. (1955): Principles of Animal Virology, New York: Academic Press Inc., p. 34.
Burnet, F. M., and Lush, D. (1938): Austral. J. exp. Biol., 16, p. 261.
Burnet, F. M., and Lush, D. (1939): J. Path. Bact., 48, p. 275.
Cairns, H. J. F. (1957): Virology, 3, p. 1.
Chu, C. M. (1948): J. Hyg. (Camb.), 46, p. 42.
Craigie, J., and Wishart, F. O. (1934): J. exp. Path., 15, p. 390.
Donnelley, M. (1951): Austral. J. exp. Biol., 29, p. 137.
Fenner, F. (1947): *Ibid.*, 25, p. 257.
Fenner, F. (1948): J. Path. Bact., 60, p. 529.
Fenner, F. (1949): J. Immunol., 63, p. 341.
Giradi, A. J., Allen, E. G., and Sigel, M. M. (1952): J. exp. Med., 96, p. 233.
Gostling, J. V. T., and Bedson, S. P. (1956): J. exp. Path., 37, p. 434.
Heinmets, F., and Golub, O. J. (1948): J. Bact., 56, p. 509.
Henle, W. (1949): J. exp. Med., 90, p. 1.
Henle, W. (1953): Advances in Virus Research. New York: Academic Press Inc., 1, p. 142.
Hoyle, L. (1950): J. Hyg. (Camb.), 48, p. 277.
Isaacs, A., and Bozzo, A. (1951): Brit. J. exp. Path., 32, p. 325.
Lawrence, J. J. (1957): Austral. J. exp. Biol., 35, p. 179.
Lépine, P., Wielgosz, G., and Reinié, L. (1951): Ann. Inst. Pasteur., 81, p. 77.
Liu, O. C., and Henle, W. (1951): J. exp. Med., 94, p. 269.
Maitland, H. B., and Tobin, B. M. (1956): J. Hyg. (Camb.), 54, p. 102.
McNair Scott, T. F., Coriell, L. L., Blank, H., and Gray, A. (1953): J. Immunol., 71, p. 134.
Metcalf, D. (1955): Austral. J. exp. Biol., 33, p. 45.
Modi, N. L., and Tobin, J. O'H. (1954): Brit. J. exp. Path., 35, p. 595.
Nossal, G. J. V., and de Burgh, P. M. (1954): J. gen. Microbiol., 10, p. 345.
Oya, A. (1955): Jap. J. med. Sci. Biol., 8, p. 399.
Pizzi, M. (1950): Hum. Biol., 22, p. 151.
Shaffer, M. F., and Enders, J. F. (1939): J. Immunol., 37, p. 383.
Siim, J. Chr. (1949): Acta med. Scand. Suppl. 234, p. 304.
Stone, J. D. (1948): Austral. J. exp. Biol., 26, p. 49.
Swain, R. H. A. (1955): Brit. J. exp. Path., 36, p. 507.
Tobin, J. O'H. (1954): *Ibid.*, 35, p. 358.
Wildy, P. (1954): Austral. J. exp. Biol., 32, p. 605.
Wildy, P., and Holden, H. F. (1954): *Ibid.*, 32, p. 621.

GENETIC SUPPRESSION OF VIRULENCE IN THE INFLUENZA VIRUS STRAIN WS—

by PATRICIA E. LIND AND F. M. BURNET[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(*Accepted for publication 25th November, 1957.*)

In a recent paper (Burnet and Lind, 1957), the properties of an influenza virus mutant, M+d, were described. M+d, derived from the strain M+, lacks the virulence for chick embryos and for mouse lung characteristic of M+; it is more heat labile and its haemagglutinin shows a different pattern of behaviour towards certain mucoid inhibitors. However, passage at low dilution, either in the allantoic cells of the chick embryo or in the de-embryonated egg, revealed a high rate of reversion of M+d to M+ with full restoration of the virulence characters of M+. Since it seemed unreasonable to postulate a genotypic loss of virulence and its recovery by back mutation and growth in a neutral environment, the hypothesis of a "suppressor gene" was advanced to account for the findings. In consequence of the results it was decided to investigate the behaviour of aWS—, a mutant of the WS— strain which had lost the pathogenicity for mouse lung characteristic of WS—, to see if this change in character were due to a complete mutation to avirulence or if it, too, could be more readily explained in terms of the suppressor gene concept. The findings are reported in this paper.

MATERIALS AND METHODS.

Virus Strains. WS–, the strain from which aWS– was derived, was first described by Burnet and Lind (1952). In the course of an investigation of the development of incomplete virus, WS– was given a number of serial passages in the de-embryonated egg. Limit infecting dilution titration in the allantoic cavity of the harvest from the second de-embryonated egg passage yielded, at limit infecting dilution, three positive eggs out of the six inoculated. Allantoic fluids from two of these eggs showed the typical *in vivo* and *in vitro* behaviour of WS–; the third was similar in all respects except that it completely lacked the WS– characteristic of high pathogenicity for the mouse lung, even after inoculation of ten times the standard test dose. This strain, termed avirulent WS– or aWS–, was taken through a number of limit infecting dilution passages in the allantoic cavity and was found to maintain its character on passage. Second and fifth limit dilution passage stocks were used to initiate the experiments described below.

The full characteristics of the WS– strain have previously been described in detail

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

(Burnet and Lind, 1952) and only that of mouse lung pathogenicity, relevant to the present study, will be mentioned here.

Determination of mouse lung pathogenicity (MLP). Virus fluids were diluted to one agglutinating dose (AD), i.e. to a haemagglutinin (HA) titre of 1 per 0·25 ml., in saline containing 10 p.c. normal inactivated horse serum. 0·05 ml. amounts of this dilution were inoculated intranasally into groups of 4 to 8, 4 week old mice under light ether-chloroform anaesthesia. The mice were observed for seven days and all deaths recorded. The surviving mice were killed and their lungs examined for areas of consolidation. Scores were made as follows: death on the fifth day or earlier scored 5, on the sixth day 4·5 and on the seventh day 4. Lesions in the surviving mice were scored 0-3·9 according to the degree of consolidation.

Inoculation of WS– under these conditions invariably caused the death of all mice between the fourth and seventh days after infection, the scores ranging from 4 to 5. Inoculation of aWS– not only failed to kill mice, but completely failed to produce any areas of lung consolidation even when the test dose was increased to 10AD. With strains of intermediate virulence the findings were less regular and, as will be noted in the protocols, disconcerting irregularities were sometimes encountered amongst a group of mice all receiving the same inoculum. To a large extent this appeared to be due to the relatively rapid diminution in the susceptibility of mice during the fourth and fifth weeks of life to the lesion producing power of intermediate strains. Direct comparison of four and five week old mice inoculated with two intermediate fluids showed average scores of 0·8-0·2 and 2·3-1·3 respectively.

Preparation of mouse lung extracts. Mice were killed by over-anaesthetization and the lungs removed aseptically, ground with alundum, suspended in 1 ml. saline per lung set and the supernatant fluid, after light centrifugation, used as starting material for further passage.

Growth of virus in the de-embryonated egg. The technique described by Lind and Burnet (1957) was used.

Limit infecting dilution (LD) titration. Serial tenfold dilutions of virus were prepared in chilled horse serum saline and 0·05 ml. amounts of the appropriate dilutions inoculated allantoically into 11 day old chick embryos. After two days' incubation at 35° C. the embryos were tested for the presence of virus in the allantoic fluid and negative eggs were returned for a further day's incubation. Infectivity titres were calculated by the method of Reed and Muench (1938). In certain cases positive allantoic fluids from embryos which had received the smallest infective inoculum were used for further passage and these are referred to as limit dilution or LD fluids.

EXPERIMENTAL.

Serial passage of aWS— at low dilution. In the case of M+d, serial allantoic passage at low dilution (10^{-2}) proved an effective means of revealing the reversion to M+, whereas passage at limit infecting dilution gave only pure lines of the M+d type. It was considered probable that under the conditions of low dilution passage the M+ revertants, although constituting only a minority of the virus particle population, were able to overgrow the slower growing M+d component. Serial low dilution passage of aWS— was therefore begun to see if mouse virulent WS— revertant strains would appear. Second limit dilution passage aWS— was diluted to 1 AD and inoculated allantoically into four chick embryos and intranasally into six mice. Allantoic fluids harvested from the eggs 40 hours later were pooled, checked for *in vitro* WS— characters, and again

inoculated at 1 AD dilution into further groups of chick embryos and mice. Six successive allantoic passages were made in this way (Series A), the dose level being kept at 1 AD (10^{-2}-10^{-3} dilution) and groups of mice and chick embryos being inoculated with virus from each passage. The MLP scores, which are shown in Table 1, show a detectable level of pathogenicity after two passages followed by a sharp increase with successive passage until full mouse virulence is attained after five passages.

TABLE 1.

Mouse lung pathogenicity of serial low dilution passages of aWS— commenced with 2nd limit dilution passage material (Series A).

Passage No.	Mouse lung pathogenicity Scores of individual mice	Average scores
1A	0 0 0 0 0 0	0·0
2A	0 0 0 0 0 0 0 0·4	<0·1
3A	0·5 2·2 1·5 0·8	1·3
4A	1·0 2·0 2·2 1·0	1·6
5A	All mice dead on 5th day	5·0
6A	All mice dead on 5th day	5·0

A second series (Series B) of low dilution passages of aWS— was carried out in the same way, beginning with fifth limit dilution passage virus. These results are shown in Table 2.

TABLE 2.

Mouse lung pathogenicity of serial low dilution passages of aWS— commenced with 6th limit dilution passage material (Series B).

Passage No.	Mouse Lung Pathogenicity Scores of individual mice				Average Scores
1B	0	0	0	0	0
2B	0	0	0	0	0
3B	0	0	0	0	0
4B	0·1	0·2	0·3	0·2	0·2
5B	1·0	1·4	1·6	0	1·0
6B	0	1·8	1·0	1·2	1·0
7B	1·2	0·1	0·3	0·5	0·5
8B	4·5	3·8	1·0	3·7	3·3
9B	2·0	2·2	3·0	2·2	2·4
10B	0·1	0·7	1·5	0·4	0·7
11B	1·2	3·2	1·4	1·2	1·8
12B	...	2·5	2·5	3·5	2·8

Comparison of the two sets of results reveals a more gradual emergence of mouse pathogenicity in Series B, full activity not being reached even after 12 passages.

Three interpretations are possible.

The results in Series A suggest the emergence of a mouse pathogen with a faster growth rate in allantoic cells than that of aWS— which it quickly over-

grows. Those in Series B might be explained by the development of mouse pathogenic virus with the same capacity for allantoic growth as aWS— so that a mixture of aWS— and WS— is maintained on passage. Each harvest would have a mouse lung pathogenicity proportional to its WS— content.

Secondly, the results could be explained by the occurrence of reversion in two or more mutational steps each represented by strains of increasing levels of mouse virulence and the full mutational change occurring more rapidly in the first passage series than in the second.

In the third place, strains of intermediate levels of mouse virulence might arise by recombination between aWS— and fully virulent WS— revertants.

To gain further insight into the changes in mouse lung pathogenicity observed during the two passage series, limit infecting dilution titrations were carried out with harvests 1 to 5 of Series A and 4, 5, 6, 8, 11 and 12 of Series B. The virus was diluted in tenfold steps and 10 to 15 chick embryos inoculated allantoically with each dilution over the limit infecting dilution range. All fluids harvested from positive eggs receiving one 50 p.c. egg infectious dose (EID_{50}) or less were submitted to mouse test. The average MLP scores given by each of these fluids is shown in Table 3.

TABLE 3.

Mouse lung pathogenicity of clones obtained by limit infecting dilution titration of low dilution passage harvests.

Low Dilution Passage No.	No. of Clones Tested	Average scores of groups of 4 mice inoculated with each fluid
1A	6	0 0 0 0 0 0
2A	7	0 0 0 0·2 0·3 0 0
3A	5	0 0 0 0 0
4A	8	0 4·0 0·2 0 2·5[1] 3·6 4·3 1·2[2]
5A	6	3·8 3·9 3·3 3·4 4·2 4·2
4B	7	0 0·1 0 0 0 0 0
5B	7	2·4[3] 1·1* 0 0·9[4] 0·3[5] 0[6] 0·7
6B	3	2·2[7] 2·5[8] 0·1
8B	10	0·3 0·2 0·3 0·9 0·4 1·4* 1·9* 1·2 1·4* 0·4
11B	6	2·4* 1·0* 1·0* 3·0 0·2 0·7
12B	5	1·3* 1·6 1·0 1·5* 2·9

Fluids giving MLP scores marked 1-6 further examined by a second limit dilution titration and mouse test of the limit positive fluids (see Text and Table 4).

7-8. The lungs were removed from these mice, titrated in chick embryos and the positive fluids tested in mice (see Text and Table 5).

* See Text.

In a number of cases in series B the average scores shown in Table 3 were difficult to assess as there was often a lack of uniformity in the scores shown by individual mice of the four inoculated with each fluid. The most frequent irregularity was the death of one of the four mice with complete lung consolidation between the 5th and 7th day after inoculation, while the remaining three de-

veloped only minor lesions and gave scores ranging from 0-1·0. The same irregularity was often found on repeating the test. Such groups are marked with an asterisk in Table 3.

Six limit dilution fluids giving intermediate MLP scores were given one or more limit dilution passages and the positive fluids obtained at or above the EID_{50} level also tested in mice. These fluids are marked 1-6 in Table 3 and the MLP scores of the limit dilution fluids obtained from them are shown in Table 4.

TABLE 4.

Mouse lung pathogenicity of limit dilution fluids obtained by titration of fluids marked 1-6 *in Table* 3.

Fluid No. (Origin)	MLP Score	Limit Dilution Passage No.	No. of Fluids Tested	Average MLP Score of Groups of 4 Mice Inoculated with Each Fluid				
[1](4A)	2·5	1	4	3·8	3·8	3·8	4·2	
[2](4A)	1·2	1	3	0·2*	0·3	0·1		
		2	3	0·4[9]	0·1[10]	<0·1[11]		
[3](5B)	2·4	1	4	0·8	0·5	0·3	0·8	
[4](5B)	0·9	1	2	0	1·6			
[5](5B)	0·3	1	2	1·2	0·5			
[6](6B)	0·8[1]	1	5	2·5	0·2	0·9	3·0	0·6

Fluid giving MLP score marked * used to initiate next LD passage.

9-11. The lungs were removed from these mice, inoculated into chick embryos and the fluids obtained tested in mice (see Text and Table 5).

1. This fluid originally gave an MLP score of 0 (see Table 3). However on retesting prior to this series of experiments it gave an MLP score of 0·8 which is recorded here.

TABLE 5.

MLP scores of allantoic fluids obtained by allantoic inoculation of extracts of mouse lungs labelled 7-8 *Table* 3 *and* 9-11 *Table* 4.

Chick embryo inoculum		Dilution inoculated	Average MLP scores of allantoic fluid harvests	
			Dose inoculated	
Extract of mouse lungs No.	MLP score		0·01 AD	1 AD
7	2·2	10^{-3}	0·4	2·0
8	2·5	10^{-3}	0·2	0·7
9	0·4	10^{-3}	0·2	2·5
10	0·1	10^{-3}	0·7	1·3
11	<0·1	10^{-3}	0·1	0·75[1]

1. An extract of a pool of these lungs was inoculated allantoically at 10^{-3} dilution. The harvest gave an MLP score of 4·8 at the 1 AD dose level.

Saline extracts were prepared from the infected lungs removed from the various groups of mice. These were inoculated allantoically into four chick embryos, usually at 10^{-2} dilution, and the pooled harvest tested in further mice. This procedure was carried out with the lungs giving the scores marked 7-8 in Table 3 and 9-11 in Table 4 and the results are given in Table 5.

Alternate mouse lung-chick embryo passage of aWS—. As an alternative to serial low dilution passage in the allantoic cavity a series of mouse lung-chick embryo passages of aWS— was carried out to see if, by this means, reversion to WS— could be accelerated.

The series was begun with low dilution passage material 4B which was diluted to 10AD and 1AD, the two dilutions being inoculated intranasally into six and four mice respectively. Two of the mice inoculated with the larger dose were killed two days after inoculation; extracts of their lungs were prepared in saline and the supernatant fluid titrated to LD in the allantoic cavity. An LD fluid was then titrated intranasally in mice by inoculation of 1AD $\times 10^{-4}$, 1AD $\times 10^{-3}$, 1AD $\times 10^{-2}$ and 1AD $\times 10^{0}$ dilutions. Two mice which had received the largest dose were killed two days later and an extract of the pooled lungs titrated allantoically. The series was continued in this manner for ten mouse and nine allantoic passages. All mice which were not sacrificed two days after inoculation to provide lung extracts for allantoic passage were kept for seven days then killed and their lung lesion scores determined.

TABLE 6.

Effect of alternate mouse lung-limit dilution allantoic passage on the MLP of aWS— commencing with allantoic fluid 4B.

Allantoic passage No.	Allantoic titre of mouse lung extracts	Mouse lung passage number	Average MLP scores for groups of mice inoculated with dilutions 1 AD				
			$\times 10^{-4}$	$\times 10^{-3}$	$\times 10^{-2}$	$\times 10^{0}$	$\times 10$
		1				0·2	0·5
1	$>10^{4}$	2	0	0	0·3	0·05	
2	$10^{5\cdot4}$	3	0	0	0·6	2·2	
3	$10^{5\cdot0}$	4	0	0	0	1·4	
4	$10^{4\cdot5}$	5	0·05	0·1	0·1	0·8	
5	$10^{5\cdot4}$	6	0	0	0	0·3	
6	$10^{4\cdot8}$	7	0	0·1	0·1	2·2	
7	$10^{4\cdot8}$	8	0·05	0·3	0·1	0·3	
8	$10^{5\cdot3}$	9	0	0	0·5	1·7	
9	$10^{5\cdot0}$	10	0	0	0·1	1·0	

As shown in Table 6 this procedure led only to a weak and irregular enhancement of mouse lung pathogenicity, although it is clear from the allantoic infectivity titres of the mouse lung extracts that virus multiplication took place in the mouse lung and reached a fairly constant level. This is in contrast to the behaviour of the original aWS— strain which completely fails to multiply in the mouse lung (unpublished findings). It would therefore appear that under these particular conditions of passage a revertant strain of a low level of mouse pathogenicity can be maintained in a stable form.

A similar series of experiments was undertaken in which the inoculation of the mouse lung extracts into the allantoic cavity was made at a dilution of 10^{-2} instead of at limit infecting dilution. Six allantoic fluids representing low dilution passages 2B, 5B, 6B, 7B, 10B and 11B were used. Each fluid was diluted to the 1AD level and inoculated intranasally into a group of 12 four-week-old mice. Three days later 6 of the 12 mice in each group were killed, extracts were prepared from each set of lungs and each inoculated allantoically into two chick embryos at a dilution of 10^{-2}, except those from 2B, which were all non-infective at 10^{-2} and were inoculated undiluted. Two days later the harvests from each pair of eggs were pooled, diluted to 1AD and each inoculated intranasally into four mice. These mice and the remainder of those which had received the original inoculum were kept under observation for seven days following injection. The survivors were then killed and the MLP scores assessed for each group. The results, which are shown in Table 7, show that a single low dilution, allantoic passage of the infected mouse lung extract gives rise to a fluid with a mouse lung pathogenicity level much higher than that of the original mouse inoculum. In this Table there is a clear indication of the progressive increase in potential mouse virulence in the course of the B series of low dilution passages in the allantoic cavity which is not so evident in the direct tests shown in Table 2. Revertants with the full pathogenicity of the original WS— were obtained only with the 10th and 11th passage fluids.

TABLE 7.

Effect of alternate mouse lung/low dilution allantoic passage on the MLP of various preparations from low dilution passage Series B.

Original fluid	MLP score of original fluid (1 AD inoculum)	Average MLP scores of fluids obtained by low dilution allantoic passage of mouse lung extracts prepared 3 days after inoculation of original fluid
2B	0	0 (tr)
5B	0·2	1·3
6B	0·8	3·0
7B	1·0	2·5
10B	2·3	4·8
11B	3·0	4·5

Detection of small amounts of WS— in the presence of excess aWS—. In conjunction with these experiments and to help in the interpretation of results, titrations were carried out to determine whether small amounts of WS— could be detected in the presence of a large excess of aWS—.

Tenfold dilutions of a fresh WS— allantoic fluid (HA titre 800) covering the range 1AD (1/800), 0·1, 0·01, 0·001 and 0·0001AD were prepared in (a) horse serum saline (HSS) and (b) the same diluent containing freshly harvested aWS— diluted to 1AD (1/450). Both sets of dilutions were inoculated intranasally into mice, using four mice per dilution, with the results shown in Table 8.

TABLE 8.

Detection of small amounts of WS— in the presence of excess aWS—.

Dose of WS—	Mouse lung pathogenicity scores							
	Diluent							
	HSS				HSS containing 1 AD of a WS—			
1 AD	5·0	5·0	5·0	5·0	5·0	5·0	5·0	5·0
0·1 AD	5·0	5·0	5·0	4·0	5·0	5·0	4·5	4·5
0·01 AD	4·5	4·0	3·9	3·7	4·5	4·5	4·5	4·5
0·001 AD	3·6	3·9	3·8	3·6	4·0	4·0	3·5	3·5
0·0001 AD	1·8	1·8	1·3	3·2	2·0	2·2	3·4	4·0

In a second experiment, similar sets of dilutions of WS— in (a) HSS and in (b) HSS containing, in this case, 10 AD of aWS—, were inoculated allantoically into chick embryos.

The EID_{50} of WS— could be determined directly from the results of inoculation of virus diluted in HSS. Harvests from embryos inoculated with the same range of dilutions of WS— prepared in the aWS— containing diluent were tested for the presence of WS— by intranasal inoculation into mice.

By direct titration in HSS the EID_{50} value of WS— was found to be $10^{-7.5}$; in the aWS— containing diluent it was $10^{-7.8}$.

It is clear from these findings that excess aWS— does not mask the activity of minimal amounts of WS—.

Serial passage of aWS— in the de-embryonated egg. aWS— stock, prepared from 5th limit dilution passage material, was diluted to 20AD titre and 5 ml. amounts were inoculated into each of 2 DEE. The standard procedure was followed and harvests were made seven hours after infection. The pooled harvest, which had a HA titre of 40, was diluted 1/3 and inoculated into two further DEE. Harvests were made from this second pair of DEE 24 hours after infection. The two fluids, whose titres were 8 and 10, were pooled, diluted 1/10 and inoculated intranasally into mice. Seven days later these mice were all alive and their lungs free of lesions.

Unlike M+d, aWS—, under these conditions, does not revert to the mouse virulent form.

Analysis of low dilution passage fluid 11*B*. The following experiment was designed to determine the approximate content of WS— virus of various levels of mouse pathogenicity in the low dilution passage fluid 11B. It was based on the assumption that if an inoculum containing particles of differing MLP were administered to mice, the yield of virus from the lungs two days after inoculation would be dominated by the type with highest MLP that was present in the inoculum. This could be determined by titrating the lung extract and then determining the MLP of the limit infecting dilution fluids. A series of dilutions of fluid 11B, 10 AD (1/80), 3 AD, 1 AD, 0·3 AD, 0·1 AD and 0·03 AD were inoculated intranasally into six mice. Two of the mice in each group were killed two days later and the remainder kept for assessment of the MLP scores seven days after inoculation. Extracts were prepared from the lungs of the killed mice and these were titrated to limit infecting dilution in the allantoic cavity. One or two limit dilution positive fluids from each group were tested in further mice at the 1 AD dose level. The results are shown in Table 9.

TABLE 9.

Analysis of low dilution passage fluid 11*B*.

Dose of fluid 11B inoculated into mice	MLP score (7 days)		Average MLP scores of LD fluids		
			1st LD* passage	2nd LD passage	
0·03 AD	0·6	Extracts of mouse	0·6	0·1	0·5
0·1 AD	0·8	lungs taken 2 days	1·2		
0·3 AD	0·7	after inoculation of	1·1	2·7	..
1·0 AD	2·5	11B, were titrated	1·2		
3 AD	3·8	to LD in allantoic	4·5	4·5	5·0
10 AD	4·2	sac.	4·2		

* Fluids giving MLP scores shown in this column were used to initiate the 2nd LD passage.

These results are clear cut. In the two lowest dilutions, 10 AD and 3 AD, the dominant virus had full pathogenicity for mice. Higher dilutions gave mouse lung infections which were dominated by virus of low virulence. Our interpretation is that the results in the fourth column of Table 9 can be taken as representing the MLP level of the type of virus of highest virulence present at each particular dilution level. Clearly in fluid 11B virus clones of low and intermediate virulence are present in much higher concentration than those of high virulence. The fact that fully virulent revertant WS— was present in such low concentration presumably means that it was at a disadvantage in competing with the intermediate clones in the allantoic cavity.

Recombination between aWS— and WS—. Since there was a definite possibility that strains of intermediate levels of mouse virulence might occur as the result of recombination between aWS— and the WS— strains to which it can give rise on low dilution passage, direct recombination experiments between the two strains were carried out. These have shown that strains of intermediate virulence can be obtained. One tested at two successive LD passages gave MLP values of 3·0 and 1·9. The experiments are continuing and will be reported in full later.

DISCUSSION.

These results are not easy to interpret. In series A our impression was that a sequence of revertants appeared, each of which combined (a) a higher mouse virulence and (b) a greater facility for growth in the allantoic cavity. This was confirmed by the single clone isolations from 4th and 5th low dilution passages. Tables 3 and 4 show that from the 4th passage a relatively stable intermediate with an MLP score of 1·2 was obtained. Passage of this strain did not greatly alter its pathogenicity, giving values of 0·1-0·3 and 0·1-0·4 in the 1st and 2nd allantoic passages respectively.

In series B the results are less consistent. By the 8th passage it seemed that reversion was nearly complete, but thereafter the average MLP came to a lower level. Limit dilution titration of all generations from 5B to 12B gave broadly similar results, the main feature of which was unreproducibility in the sense that occasional mice showed much more severe lesions than others receiving the same inoculum. There was also much variation from clone to clone. When virus from a lung showing lesions more marked than those of its companions was passed through the allantoic cavity, the usual result was to obtain a fluid of full WS— virulence. On the other hand, the great majority of tests from a lung showing minor lesions gave fluids with similar weak or intermediate MLP.

Our interpretation must be cautious, but we believe that there is evidence for the existence of several forms of virus of some genetic stability in addition to aWS— and WS—. It must remain open whether the intermediates represent, say, three distinct genotypes or are merely artificial selections from a continuous or at least much larger series of intermediates. We tend to favour the latter hypothesis, but since the implications are more or less independent of how many intermediate types there are we may develop the simpler case. Let us call aWS— 0 and WS— 4, and accept intermediates 1, 2 and 3. The postulated characteristics of these intermediates will need to include (a) the capacity of a pure population of the type to produce mouse lung lesions at the 1AD dose level, and (b) the types and concentrations of mutants produced during multiplication from a single infective unit to a full titre infective fluid. These are best indicated in the schematic diagrams of Figs. 1 and 2, but can also be tabulated as shown in Table 10.

TABLE 10.

Virus types postulated in process of reversion from aWS— to WS—.

Type	Direct MLP	Derivatives	Observed MLP
0(a WS—)	0	1	0
1	0	(0), 2	0·2-2·0
2	0·2-2·0	(0, 1), 3, (4)	0·2-3·5
3	2·5-3·8	(0, 1, 2), 4	2·5-4·0
4(WS—)	4·5+	...	4·5+

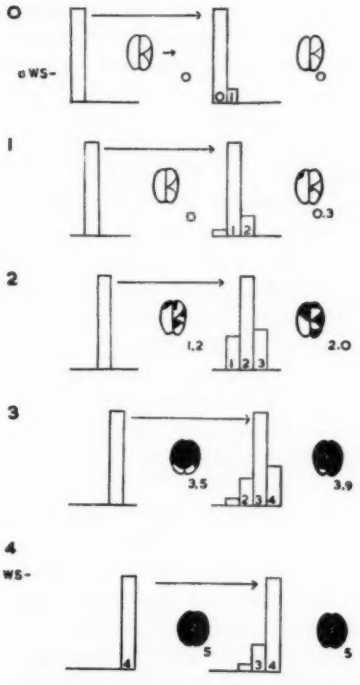

Fig. 1. To illustrate the hypothesis of intermediate genotypes between aWS— (0) and WS— (4). Each histogram represents the constitution of an infective fluid in terms of the 5 postulated types, followed by a diagram of the type of lung lesion produced in mice by 1 AD dose of the infective fluid. The first series shows the activity of the hypothetical pure types. The second the constitution and activity of the fluid resulting from inoculation of a single infective dose of the type indicated. All such fluids will be mixtures of the original with mutant types.

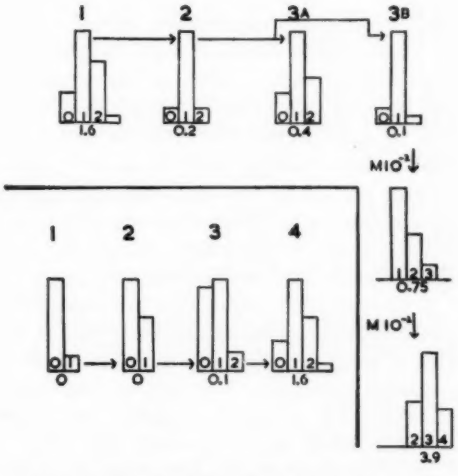

Fig. 2. To illustrate typical experimental results in terms of the hypothesis of intermediate types. The upper series of 2 limiting dilution passages in the allantoic cavity followed by two mouse passages shows the type of result obtained with derivatives of fluid 2(4A). See Tables 4 and 5. The lower series shows the common features of the A and B series of low dilution passages of aWS—. Symbols as in Fig. 1 with the mouse pathogenicity scores of a 1 AD dose of virus indicated beneath each histogram.

The MLP values in the first column of Table 10 are those assumed for a 1AD dose of a pure population of the type in question. It is further assumed that in fact by the time an allantoic fluid is harvested it will contain a proportion of mutants as well as the dominant form presumably corresponding to the genotype of the particle initiating the clone. The MLP observed (column 3) will therefore be, in most cases, the result of the activity of the most actively mouse pathogenic variant present in the fluid. This can be seen clearly in the results obtained from the analysis of fluid 8B. When tested directly this fluid gave an MLP score of 3·3, but the constituent clones when tested gave values ranging from 0·2-1·9. The actual MLP of any fluid will depend on (a) what mutations have occurred, and (b) whether admixture with excess of virus of low virulence interferes with the development of lung lesions by a small proportion of virus of higher virulence.

Factor (b) may be concerned in producing the discrepancies from one mouse to another that are observed with intermediate strains. With mixtures of 0 and 4 there is no evidence whatever of interference by very large amounts of 0 on the lesion producing capacity of a few particles of 4. This, however, does not exclude interference effects among intermediate types.

The results obtained with alternate mouse lung-limit dilution allantoic passage suggest that, by this means, a stable form of intermediate type 1 can be maintained indefinitely. When, however, the conditions are altered by transferring a low dilution of the mouse lung extract to the allantoic cavity and testing the resulting fluid, in each instance more virulent fluids are obtained (Table 7). From the 10th and 11th passages of series B a single such passage gave a fluid containing revertants of full virulence. Passages 2, 5, 6 and 7 gave lower values and the whole series suggested that with each there was an increasing probability of finding the later stages of the reversion series.

We are well aware that an interpretation of this sort is elastic enough to cover almost any combination of results and we have also been very conscious that a single contaminant particle of WS— (4) could result in an apparent complete reversion. We believe, however, that the results do represent a true picture of the complete or partial return of virulence in this unusual strain.

The results have a general resemblance to those reported in an earlier paper dealing with the mutant M+d (Burnet and Lind, 1957), but there are important differences. The change WS— to aWS— presumably was the result of a single genetic incident since the mutant was unassociated with any intermediates. The reversion to full virulence, however, required at least two, and probably four or more, genetic steps. This was confirmed by the recognition of recombination between the two extreme forms.

The process might be represented conventionally as

Wvvvv	$W\overline{vvvv}$	$W\overline{\overline{vvvv}}$	$W\overline{\overline{\overline{vvvv}}}$	$W\overline{\overline{\overline{\overline{vvvv}}}}$	Wvvvv
WS—	aWS—				WS—

where the bar represents a suppressor effect preventing the phenotypic manifestation of the virulence genes, v, which are beneath it. We do not, however, feel that such a presentation has any heuristic value. We are more interested in attempting a careful examination of the functional differences between the two extremes in the hope that this may give some clue to the real nature of the process that we have called a suppressor gene effect.

Irrespective of any theoretical interpretation, the fact that a strain may lose *all* virulence for a certain host by a genetic change which by appropriate manipulation can be completely reversed, may be highly relevant to the general problem of obtaining "fixed" attenuated variants.

SUMMARY.

The avirulent strain aWS— maintains its properties indefinitely on limit infective dilution (LD) passage in the allantoic cavity and a single low dilution passage in the allantoic cavity or the de-embryonated egg gives fluids with no trace of mouse lung pathogenicity.

With repeated passage in the allantoic cavity at low dilution, mouse virulent forms appear: there were significant differences in two such passage series, A and B, that were analysed in detail.

Series A showed the re-appearance of fully virulent WS— after five low dilution passages; from the 4th passage were isolated clones of low virulence which retained their virulence level on LD passage and clones of intermediate virulence which readily reverted to full virulence.

In series B direct passage never allowed the appearance of full virulence, and there was no marked change in character between 4th and 12th low dilution passages. Most such fluids on analysis at LD gave clones of low intermediate virulence which were stable on LD passage.

Revertants of full virulence could be obtained by a single mouse-egg passage from clones derived from the later passages of series B but not from the earlier ones.

Limited evidence of recombination between WS— and aWS— was obtained.

An interpretation of these results is attempted on the basis that the loss of virulence is a suppressor gene effect and that reversion takes place in four stages. The results obtained depend on the relative survival value of the revertant types appearing in any given virus population.

Acknowledgment. The authors wish to thank Miss Judith Gorrie for her very competent technical assistance.

REFERENCES.

Burnet, F. M., and Lind, P. E. (1952): Austral. J. exp. Biol., 30, p. 469.
Burnet, F. M., and Lind, P. E. (1957): *Ibid.*, 35, p. 225.
Lind, P. E., and Burnet, F. M. (1957): *Ibid.*, 35, p. 57.
Reed, L. J., and Muench, H. (1938): Amer. J. Hyg., 27, p. 493.

STUDIES ON INTERMEDIARY IRON METABOLISM

XII. MEASUREMENT OF THE IRON DERIVED FROM WATER SOLUBLE AND WATER INSOLUBLE NON-HAEM COMPOUNDS (FERRITIN AND HAEMOSIDERIN IRON) IN LIVER AND SPLEEN

by I. KALDOR[1, 2]

(From the New South Wales Red Cross Blood Transfusion Service, Sydney).

(*Accepted for publication 22nd November, 1957.*)

Iron is present in tissues in several different forms, as shown in Fig. 1. The haem iron includes that of haemoglobin contained in the tissue and that of the haem catalytic enzymes. The amount of iron in compounds representing inter-

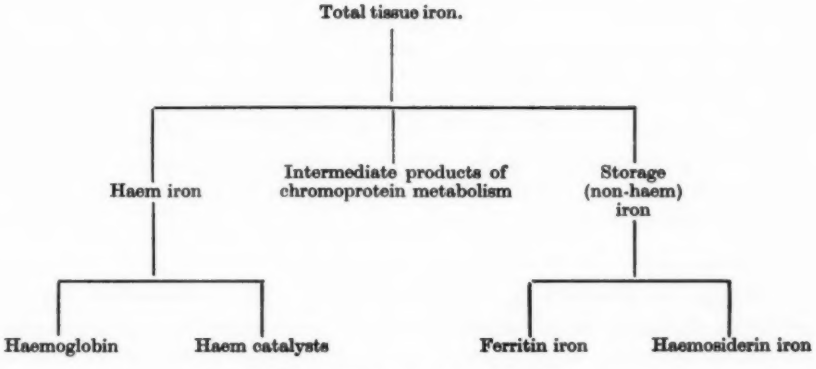

Fig. 1. The various forms of iron in tissue.

mediate products of chromoprotein metabolism is small and can be ignored in relation to overall iron metabolism. Non-haem iron compounds occur chiefly in the liver, spleen and bone marrow. They store the reserve iron of the virtually closed intermediary metabolic cycle of body iron. One storage compound, now identified as haemosiderin, has long been known as the stainable iron of tissues. Another compound, ferritin, was isolated more recently (Laufberger,

[1] This work was undertaken during tenure of a Fellowship of the National Health and Medical Research Council.

[2] Present Address: Department of Physiology, The University of Western Australia, Nedlands, W.A.

1937) and its peculiar characteristics have since been studied extensively (Granick, 1946). It is believed that ferritin and haemosiderin are the only important iron storage compounds (Drabkin, 1951). A number of physiological and pathological factors influence the amount of storage iron (Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch and Fluharty, 1950) and at least some of these factors also affect the relative proportions of iron stored as ferritin and haemosiderin (Granick, 1946; Gubler, Lahey, Chase, Cartwright, and Wintrobe, 1952; Shoden, Gabrio and Finch, 1953).

All practical methods of measuring the two storage compounds have been based on a simple difference in solubility. Ferritin is soluble in water and haemosiderin is insoluble in water but soluble in acid. The validity of using this solubility difference as the basis of separating the two compounds was proved by immuno-chemical and radiotracer techniques (Gabrio, Shoden and Finch, 1953).

In the present paper, measurements of the two fractions of storage iron will be described. Water and acid extracts of tissue were assayed for iron by a method described earlier for the measurement of total storage iron (Kaldor, 1954a). The iron derived from these extracts will be referred to as ferritin and haemosiderin iron respectively. Two relationships shown in Fig. 1 were investigated. They are:

Total tissue iron = storage iron plus haem iron. It was shown previously (Kaldor, 1954a) that the method used for storage iron measures all the non-haemoglobin iron and only a small fraction of the haemoglobin iron present in tissues. This was established by showing that the storage iron content of tissues was only slightly increased by the addition of relatively large amounts of haemoglobin to the tissue aliquots. In the present work this conclusion was confirmed by direct measurements.

Storage iron = ferritin iron plus haemosiderin iron.

Finally, the relative proportions of ferritin iron and haemosiderin iron were measured in the tissues of animals which had been subjected to various experimental procedures. In addition, the effects of some variations in technique were also tested.

Materials and Methods.

Tissues. Livers and spleens of rabbits and rats were used. The rabbits and some of the rats were normal adult animals. Other adult rats were selected from a group which had been injected intravenously with saccharated oxide of iron 12 months previously. Each rat had received 25 mg. of elemental iron. The organs were removed from all animals after exsanguination by heart puncture under anaesthesia. No attempt was made to remove the residual blood from the tissues.

In most cases tissue aliquots ranging from 0·1 to 0·6 gm. were cut and weighed either fresh or after storage in a deep freezer. These aliquots were homogenized with distilled water in a wet grinder, driven by a variable speed electric stirrer motor. The livers and spleens of the seven rats previously injected with iron were pooled and homogenized with distilled water in a Waring Blendor. Aliquots of these homogenates, each representing 0·2

gm. of spleen or 0·4 gm. of liver tissue, were stored frozen in test tubes and provided an easy means of replicating experimental material.

Total Tissue Iron. Aliquots were wet ashed with 1 ml. of concentrated sulphuric acid and 1 ml. of 70 p.c. perchloric acid. Iron was measured in the digest absorptiometrically with ortho-phenanthroline reagent, as will be described below (see Ferritin and Haemosiderin Iron (5)).

Haem Iron. Haem was measured absorptiometrically as acid haematin in the following way: Homogenized aliquots were transferred with 5 ml. of distilled water into a 25 ml. volumetric flask. The flask was made up to volume with a solution containing 80 p.c. of acetone and 20 p.c. of a 2·5 p.c. w/v solution of hydrochloric acid in distilled water. After the flask was shaken, its contents were transferred into a tube and centrifuged for 20 minutes with a force of 2000 × g. The light absorption of the supernatant solution was measured in a Unicam SP 600 spectrophotometer at 635 mμ wave length using a cuvette of 10 mm. optical path. The instrument was calibrated for acid haematin with solutions prepared from blood in the same way as described above for tissues. The haemoglobin content of the blood used in the preparation of these solutions was measured absorptiometrically by the method of Walsh, Arnold, Lancaster, Coote and Cotter (1953). Hüfner's figure (0·334 p.c.) was used for calculating the iron content of haemoglobin.

Storage (Non-Haem) Iron. This was measured as described earlier (Kaldor, 1954a).

Ferritin and Haemosiderin Iron. Homogenized aliquots were extracted with distilled water. After centrifugation the supernate and the residue were treated separately, but in essentially the same way as aliquots were treated when measuring storage (non-haem) iron. The following is a step by step account of the technique:

1. An homogenized aliquot is transferred into a volumetric flask of 25 or 100 ml. capacity, depending on the expected iron content. The flask is made up to volume with distilled water. After thorough shaking, the contents of the flask are transferred to a Lusteroid tube. The tube is centrifuged for 30 minutes with a force of 2000 × g.

2. (a) 7·5 ml. of the supernate is transferred to a 15 ml. graduated centrifuge tube. The remainder of the supernate is pipetted off the residue and discarded.

(b) The residue is resuspended in about 2 ml. of distilled water and transferred to a 15 ml. graduated centrifuge tube with distilled water to a total volume of 7·5 ml.

(c) 7·5 ml. of distilled water is pipetted into a 15 ml. centrifuge tube to serve as a blank.

3. The three tubes containing supernate, residue and distilled water respectively are then treated in like manner. 5 ml. of a 10 p.c. w/v solution of hydrochloric acid is added. After mixing, the tubes are placed in a 90° C. waterbath for 30 minutes and centrifuged for 15 minutes with a force of 3000 × g.

4. 10 ml. of each supernate is pipetted into another 15 ml. centrifuge tube. 1 ml. of a 40 p.c. solution of trichloracetic acid is added. After mixing, the tubes are placed in a waterbath and centrifuged as in (3), but the tubes are cooled in an ice bath before centrifuging.

5. Duplicate aliquots of 5 ml. of the last supernates are pipetted into graduated test tubes. Two drops of a 1 p.c. solution of para-nitrophenol indicator are added and the solution is neutralized with ammonium hydroxide until it turns pale yellow. 0·5 ml. of a pH 4·6 acetic acid-sodium acetate buffer solution is added, followed by 2 drops of a 1 p.c. solution of ortho-phenanthroline reagent. The solution is made up to 8 ml. with distilled water and mixed. After an interval of 30 minutes the light absorption of the solution, in a cuvette of 13 mm. optical path, is measured in a photoelectric colorimeter using an EEL No. 623 gelatine light filter with an absorption maximum at 490 mμ wave length.

The iron content of the supernate and of the residue of the tissue homogenate, representing ferritin and haemosiderin iron respectively, is calculated from the instrument readings, allowance being made for the iron content of the blank solution.

The procedure in paragraph 5 above is similar to that described for measuring the serum iron value in an earlier paper (Kaldor, 1953). Details of the photoelectric colorimeter and its calibration for iron were given in this earlier paper.

RESULTS.

Table 1 shows the results of the analyses performed on a rabbit liver and spleen. The sum of the figures obtained for storage iron and haem iron exceeds the figure obtained for total tissue iron by 1 p.c. in the case of the liver and by 6 p.c. in the case of the spleen. These differences are not statistically significant, but they are probably due to the fact that a small amount of haemoglobin iron is included in the measurement of storage iron. In this regard it should be noted that the haem iron in the spleen is ten times that in the liver.

TABLE 1.

Measurements of the various iron fractions in rabbit liver and spleen.

	Liver		Spleen		Iron content of fraction as a percentage			
					of total tissue iron		of storage iron	
	Number of replicate aliquots	Iron mg. per gm. tissue (mean ± std. error)	Number of replicate aliquots	Iron mg. per gm. tissue	Liver	Spleen	Liver	Spleen
Total tissue iron	5	0·31 ±0·007	4	1·44 ±0·100	—	—	—	—
Storage iron	6	0·29 ±0·009	4	1·30 ±0·072	94	90	—	—
Haem iron	4	0·023 ±0·003	3	0·23 ±0·010	7	16	—	—
Ferritin iron	6	0·24 ±0·004	4	0·60 ±0·015	—	—	83	46
Haemosiderin iron	6	0·057 ±0·002	4	0·71 ±0·030	—	—	20	55

The sum of the ferritin and haemosiderin iron is almost the same as the storage iron of both tissues. There is, however, a significant difference in the relative proportions of the two components in the tissues, ferritin iron constituting a greater part of the storage iron in the liver than in the spleen.

Effect of some Variations of Technique on Results.

The effect of variations of the saline concentration during extraction of the ferritin iron was investigated to determine whether this influenced the proportion of iron found in the extracts. The concentration of sodium chloride was

varied between 0·056 p.c. and 0·90 p.c., but neither the ratio of iron in the two extracts nor the total amount of iron extracted was altered.

Determinations were made on aliquots obtained from liver preserved under different conditions. The purpose was to ascertain whether the method used for preparing homogenates was important. Four replicate measurements were made on (a) homogenates of freshly obtained aliquots; (b) homogenates prepared from freshly obtained aliquots but stored for several weeks in the frozen state before analysis; and (c)homogenates prepared from tissues which had been frozen for some time. It was found that these variations in preparation did not affect the results.

The effect was investigated of varying the centrifugal force applied for separating the water insoluble fraction. Gabrio *et al.* (1953) found that it was possible to change the proportion of iron in the two fractions by varying the speed of centrifugation. In the present work the centrifugal force ranged between 500 and 10,000 × g., but the results were not changed.

Effect of Age, Sex and Supplementary Iron Feeding on the Distribution of Iron in Rats.

In another paper (Kaldor and Powell, 1957) the effect of sex and age on the storage of iron in the liver and spleen of rats was described. Briefly, it was found that at all ages females stored more iron than males and in both sexes the amount stored increased progressively with age. When the diet was supplemented with iron the total amount of storage iron increased. The organs from

TABLE 2.

Ferritin and haemosiderin iron in the livers and spleens of rats.

Age in Months	Sex	Normal Diet				Period of feeding in months	Supplemented Diet			
		Livers		Spleens			Livers		Spleens	
		Storage iron mg. per gm.	Percentage ratio of FN Fe to HSN Fe*	Storage iron mg. per gm.	Percentage ratio of FN Fe to HSN Fe		Storage iron mg. per gm.	Percentage ratio of FN Fe to HSN Fe	Storage iron mg. per gm.	Percentage ratio of FN Fe to HSN Fe
4	Male	0·19	77/23	0·49	37/63	2	0·40	68/32	0·64	48/52
	Female	0·37	73/27	0·87	44/56		0·68	71/29	0·93	42/58
8	Male	0·18	86/14	0·96	53/47		0·51	83/17	2·40	47/53
	Female	0·55	80/20	2·14	74/26	3	1·09	80/20	2·91	51/49
18	Male	0·24	79/21	0·90	75/25	3	0·73	64/36	3·54	73/27
	Female	0·68	84/16	2·21	74/26		1·68	70/30	3·59	69/31

* In this and in subsequent tables ferritin iron is designated by FN Fe and haemosiderin iron by HSN Fe.

the animals used in these experiments were preserved in the frozen state and later were assayed for ferritin and haemosiderin iron. Approximately equal parts of the livers were homogenized together and the total storage iron taken to be the sum of the iron in the two fractions.

The results of these assays are shown in Table 2, which also gives details of the experiments. The ratio of ferritin iron to haemosiderin iron in the livers is not consistently altered by age, sex or supplementary feeding and consequently does not vary with the total storage iron content. On the other hand, the percentage of the ferritin fraction in the storage iron of the spleen increases significantly in the older animals. This occurs in animals of both sexes, but is not related to the total amount of storage iron. For example, male animals 18 months old with a mean iron content of 0·90 mg. per gm. of splenic tissue had approximately the same percentage of the iron in the ferritin fraction (75 p.c.) as did male animals which received the supplemented diet and had 3·54 mg. of iron per gm. of tissue. It is also of interest that these older animals had a similar percentage of iron in the spleen as ferritin as had animals of all ages in the liver.

Effect of Injections of Saccharated Oxide of Iron on the Distribution of Iron in the Liver of Mice.

It was found (Kaldor, 1955b) that following intravenous injection of iron in this form, the non-haemoglobin iron content of the liver showed a large immediate increase followed by a gradual decrease. Only about half of the iron initially deposited in the liver remained in this organ ten weeks after the injection. Intraperitoneally injected saccharated oxide of iron was precipitated and phagocytosed within the peritoneal cavity. The precipitated material persisted in the peritoneal membranes for several weeks and produced a gradual increase in the iron content of the liver. In the report of this experiment a constant dose of 40 mg. of iron per kilogram of body weight was used. The livers of the animals injected in this way were frozen and in addition those from animals injected with double the dose were available.

Portion of each liver had been used for the previous analyses. The remaining parts were homogenized together and assayed for ferritin and haemosiderin iron, with the results shown in Table 3. The storage iron concentration shown is the sum of the two fractions and not the result of a direct analysis. Following intravenous injection some of the saccharated oxide of iron passes to the liver and is recovered in both the ferritin and the haemosiderin fractions. It can be calculated that at the end of the first week the ferritin iron fraction had increased from 0·09 mg. per gm. of tissue in the controls to 0·54 mg. per gm. in the animals injected with 40 mg. iron per kilogram of body weight, and the haemosiderin iron fraction from the control value of 0·02 mg. to 0·48 mg. in the injected animals. During the following weeks the reduction in the total storage iron is associated with a proportionate decrease in the haemosiderin iron

TABLE 3.

Ferritin and haemosiderin iron in the liver of mice injected with saccharated oxide of iron.

Interval between injection and killing in weeks	Dose mg. Fe per Kg. body weight	Mice injected intravenously		Mice injected intraperitoneally	
		Storage iron in liver mg. per gm.	Percentage ratio of FN Fe to HSN Fe	Storage iron in liver mg. per gm.	Percentage ratio of FN Fe to HSN Fe
Controls	nil	0·12	81/19	0·12	81/19
1	40	1·01	53/47	0·17	70/30
	80	1·28	67/33	0·31	70/30
2	40	0·66	70/30	0·20	73/27
	80	1·20	69/31	0·35	67/33
4	40	0·52	69/31	0·20	77/23
	80	0·93	73/27	0·42	76/24
10	40	0·45	73/27	0·27	80/20
	80	0·75	74/26	0·46	69/31

fraction, but the absolute amounts are reduced in both fractions. A similar pattern is seen in the livers of animals injected with twice the amount of iron.

Following intraperitoneal injection there is, by contrast, a gradual increase in the total storage iron of the liver. As with intravenous injection the increase involves both the ferritin and the haemosiderin fractions but the haemosiderin to a slightly greater extent in the early stages.

Saccharated oxide of iron is precipitated by body fluids and the precipitated material is insoluble in water but yields its iron to acid extraction (Kaldor, 1955b). When it is injected intravenously the precipitate is taken up by the reticulo-endothelial cells in the liver and elsewhere in the body and the bulk of the iron rapidly released to form both ferritin and haemosiderin. The remainder, still in the saccharated form, will be extracted in the haemosiderin fraction. Thus, shortly after injection the haemosiderin fraction will appear to be relatively high, but will gradually decrease as the iron is released from the remaining saccharated form. Following intraperitoneal injection the bulk of the material is precipitated in the peritoneal cavity and phagocytosed locally. Gradual release of the iron increases the total storage iron of the liver between one and ten weeks after injection. At the time of injection a small part of the dose may escape into the blood stream. This will pass to the liver and may explain the relatively greater amount of the haemosiderin fraction found in the early stages.

The Effect of Experimental Anaemia on the Distribution of Iron in the Liver.

It was shown earlier (Kaldor, 1954b) that the storage iron of the liver and spleen of rats increases when haemolytic anaemia is produced for some weeks

with phenylhydrazine hydrochloride. The rats were allowed a period of recovery of at least 4 weeks after the last injection of phenylhydrazine. They were then killed and the tissues analysed. The enhancing effect of anaemia on the absorption of iron from the intestines was found in rats fed on a normal diet and also in rats when the diet was supplemented with iron. Preliminary splenectomy did not influence the amount of iron absorbed but in splenectomised rats the amount of iron found in the liver was equal to that found in the liver and spleen combined in intact rats under the same conditions (Kaldor, 1955a). The livers of all the rats used in these experiments were available for assay of the iron fractions. Approximately equal parts of each liver were homogenized together and replicate estimations were made of the ferritin and haemosiderin iron. The mean results are shown in Table 4.

TABLE 4.

Ferritin and haemosiderin iron in the livers of rats made anaemic with phenylhydrazine.

Group	Treatment	Storage iron in livers mg. per gm.	Percentage ratio of FN Fe to HSN Fe
1	Controls—intact	0·72	82/18
2	Controls—splenectomised	0·93	81/19
3	Phenylhydrazine anaemia—intact rats	1·61	73/27
4	Phenylhydrazine anaemia—splenectomised rats	2·49	73/27
5	Phenylhydrazine anaemia—intact rats on a diet supplemented with iron	2·71	70/30
6	Phenylhydrazine anaemia—splenectomised rats on a diet supplemented with iron	4·88	62/38

Compared with the control groups all the treated groups showed an increase in the proportion of haemosiderin iron. In general the proportion increased as the total storage iron increased. However, the additional iron was found in both fractions.

DISCUSSION.

It is emphasized that in this work it has not been demonstrated that the iron measured in the water soluble fraction is derived exclusively from ferritin. Similarly, the water insoluble fraction may not represent only haemosiderin iron. However, Gabrio *et al.* (1953) who used the same principle in the separation of the two fractions demonstrated with immunochemical and radiotracer techniques that ferritin and haemosiderin were localised in the water soluble and water insoluble fractions respectively. In the present work, it is known that, within the limits of experimental error, the difference between the total iron and the haem iron of the tissues is equal to the sum of the iron derived from the water soluble and the water insoluble/acid soluble non-haem compounds.

The distribution of storage iron between the ferritin and haemosiderin fractions of the liver is similar in the three species of animals used, *viz.*, rabbit, rat

and mouse. In rats the ratio is not determined by the age or sex of the animals nor by an increase in the total storage iron produced by supplemented iron feeding. When the total storage iron is increased during phenylhydrazine induced anaemia, the additional iron is found in both fractions, but the percentage in the haemosiderin fraction is slightly greater. Intravenous injections of saccharated oxide of iron in mice produced an increase in both fractions, but to a greater extent in the haemosiderin than in the ferritin fraction. The normal proportions were restored some weeks after injection when the total storage iron in the liver had decreased.

The pattern in the spleen of the rat and rabbit differs from that in the liver, the haemosiderin iron representing a greater proportion of the storage iron. In the spleen of young rats the haemosiderin iron is greater than the ferritin iron, but this is reversed in older animals.

No explanation can be offered for the organ differences or for the different ratios in the liver when the storage iron is increased in various ways. However, it can be concluded that excess iron is stored in both the ferritin and haemosiderin complexes in the liver and spleen. The iron content of both ferritin and haemosiderin has been shown to vary within fairly wide limits (Granick, 1946) and it is therefore possible that an increase in the iron of either of the two fractions may not be associated with a parallel increase in the protein moieties of these compounds.

SUMMARY.

A method is described for measuring the iron of the water soluble and water insoluble fractions of tissues. It is assumed that these fractions contain the ferritin and haemosiderin iron respectively. Generally, more iron is stored in the ferritin fraction of the liver than in the haemosiderin and this applies to the three species of animals investigated. The reverse applies in the spleen of young rats but not in the spleen of older rats. The ratio of ferritin iron to haemosiderin iron has also been determined in the organs of animals when the total storage iron has been increased by various treatments.

Acknowledgments. The author is indebted to Dr. R. Lemberg, F.R.S., for advice regarding the measurement of total haem content of tissue.

REFERENCES.

Drabkin, D. L. (1951): Physiol. Rev., 31, p. 345.
Finch, C. A., Hegsted, M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S., and Fluharty, R. G. (1950): Blood, 5, p. 983.
Gabrio, B. W., Shoden, A., and Finch, C. A. (1953): J. biol. Chem., 204, p. 815.
Granick, S. (1946): Chem. Rev., 38, p. 379.
Gubler, C. J., Lahey, M. E., Chase, M. S., Cartwright, G. E., and Wintrobe, M. M. (1952): Blood, 7, p. 1075.
Kaldor, I. (1953): Austral. J. exp. Biol., 31, p. 41.

Kaldor, I. (1954a): *Ibid.*, 32, p. 795.
Kaldor, I. (1954b): *Ibid.*, 32, p. 801.
Kaldor, I. (1955a): *Ibid.*, 33, p. 637.
Kaldor, I. (1955b): *Ibid.*, 33, p. 645.
Kaldor, I., and Powell, Marjorie (1957): *Ibid.*, 35, p. 123.
Laufberger, V. (1937): Bull. Soc. Chim. biol., 19, p. 1575. Quoted by Granick, S. (1946): Chem. Rev., 38, p. 379.
Shoden, A., Gabrio, B. W., and Finch, C. A. (1953): J. biol. Chem., 204, p. 823.
Walsh, R. J., Arnold, Barbara J., Lancaster, H. O., Coote, Margaret A., and Cotter, Helen (1953): A Study of Haemoglobin Values in New South Wales. N.H. and M.R.C. Special Report Series No. 5, Canberra.

THE EFFECT OF INSULIN ON THE METABOLISM OF MAMMARY GLAND SLICES FROM HYPOPHYSECTOMIZED RATS

by T. R. BRADLEY[1]

(From the Department of Physiology, University of Melbourne).

(*Accepted for publication 10th December, 1957.*)

Slices of mammary gland tissue from lactating rats are sensitive to insulin *in vitro*, treatment with low concentrations of insulin giving rise to substantial increases in the respiratory quotient, uptake of glucose from the incubation medium and fatty acid synthesis by the slices (Balmain, French and Folley, 1950; Balmain and Folley, 1951; Balmain, Folley and Glascock, 1954).

Hypophysectomy of lactating rats has been shown to inhibit the glucose uptake of mammary gland slices (Bradley and Cowie, 1956), a response which is the reverse of that observed in diaphragm muscle after removal of the pituitary (Krahl and Park, 1948).

In view of the effects of insulin on normal lactating mammary gland metabolism and the severe and immediate effects of removal of the pituitary on this tissue, the response to insulin, *in vitro*, of mammary gland slices from lactating rats after hypophysectomy has been investigated.

Methods.

Hooded rats fed *ad lib.* were used at the 14th to 16th day of lactation. Hypophysectomy was performed by the usual parapharyngeal approach, the fossa being traversed with a fine sucker after removal of the pituitary in an attempt to ensure complete absence of small remnants of tissue. Hypophysectomized animals resumed feeding shortly after the operation when fresh greens were added to the diet. Effectiveness of the operation with respect to the cessation of lactation was checked by observation of the absence of milk in the abdomen of the pups, despite the administration of oxytocin [Pitocin (Parke-Davis), 1 unit twice daily intraperitoneally], and observed suckling motions by the pups. Further check of the completeness of removal of pituitary tissue was made at autopsy by careful inspection of the fossa before removal of the mammary glands for the experiment.

Mammary glands were excised, sliced with a microtome (Stadie and Riggs, 1944) to give slices of approximately 0·4 mm. thickness (Folley and French, 1949). The slices were then washed twice in cold saline bicarbonate medium (Krebs and Henseleit, 1932) gassed with 95 p.c. O_2 : 5 p.c. CO_2 mixture, without added substrate. Samples of 100 mg. of slices

[1] Working with a full-time grant from the Anti-Cancer Council of Victoria.

(weighed after removal of excess fluid) were placed in conventional Warburg flasks and 2·0 ml. of cold incubation medium (as above) containing 0·3 p.c. glucose, with or without insulin as indicated in the text, was added; 0·2 ml. of 3N HCl. was placed in the sidearms of the flasks. The flasks were gassed in the bath at 37° C. for 10 minutes and equilibrated for a further 5 minutes.

Composite respiration values were recorded (Elliott, Greig and Benoy, 1937). The glucose content of samples of the media from flasks was measured by the anthrone method (Morris, 1948; Seifter, Dayton, Novic and Muntwyler, 1950; Prohkovnik and Nelson, 1953) and lactic acid was measured by the Barker and Summerson (1941) method.

Three replicates of each of the treatments, i.e. control (no insulin), 0·5 µg./ml. insulin,[2] 2·5 µg./ml. insulin were used in each experiment. These concentrations of insulin were chosen as being minimal and maximal effective doses estimated from preliminary experiments. Treatments were randomized to flasks. At the start of the experiment acid was tipped in three flasks to give zero time glucose and lactic acid contents in the media. After two hours incubation acid was similarly tipped in the experimental flasks. Normal, 24-hour and 48-hour hypophysectomized rats were used at random to prevent any possible bias with respect to alterations in the potency of the stock insulin solution from which dilutions were made before each experiment.

Estimation of the significance of treatments was made by using an analysis of variance technique in which the interaction "rats × treatments" mean square term was used as the denominator in computing variance ratio values (Goulden, 1952). This mean square term was then used for computation of the standard error of difference of means for subsequent "t" tests between individual treatments.

RESULTS.

The responses obtained with mammary gland slices from normal lactating rats showed clearly the effect of insulin, *viz.*, significant increases in composite respiration and glucose uptake even with the lower concentration. Significant increases in lactic acid production were regularly observed with the higher concentration of insulin.

Control mammary tissue taken from rats 24 hours after hypophysectomy showed markedly reduced glucose uptake and composite respiration and increased lactic acid production values compared with those obtained with normal lactating mammary tissue. In the presence of the lower concentration of insulin slight but not significant increases in glucose uptake values were found; lactic acid production and composite respiration values were practically identical with those of the control tissue. With the higher insulin concentration there were significant increases in glucose uptake, lactic acid production and composite respiration. Inspection of the results obtained with tissue from individual rats shows that in some the lower insulin concentration was effective, but in others totally ineffective, whereas the higher concentration was more regularly effective.

Control tissue taken 48 hours after hypophysectomy showed the same types

[2] Insulin used was made by Commonwealth Serum Laboratories, Victoria; approximately 20 i.u./mg. insulin activity and essentially free of glucagon activity.

of changes as seen in the 24 hours after hypophysectomy group. A small but significant (due to the regularity of the response) increase in composite respiration was observed with the higher insulin concentration, but neither of the insulin concentrations used produced significant differences in glucose uptake or lactic acid production values.

TABLE 1.

Glucose uptake by mammary gland slices (μg./100 mg. moist weight tissue/120 minutes.)

Rat No.	Normal Lactating			Hypophysectomized 24 hrs.			Hypophysectomized 48 hrs.		
		Insulin			Insulin			Insulin	
	Control	0·5 μg./ml.	2·5 μg./ml.	Control	0·5 μg./ml.	2·5 μg./ml.	Control	0·5 μg./ml.	2·5 μg./ml.
1	1800*	1950	2970	413	657	703	343	260	377
2	1460	1960	2767	320	247	740	397	490	613
3	1763	1967	3090	367	397	463	420	467	460
4	1453	1777	2000	217	427	480	220	190	317
5	2190	2803	3427	487	430	533	140	197	140
Means	1733	2091	2851	361	431	584	304	321	381
Variance Ratio†	F = 36·02; P<·001			F = 4·78; P<·05>·01			F = 2·50; P>·05		
"t" tests	Insulin 0·5μg/ml. v. control "t" = 2·65; P<·05>·01			Insulin 0·5μg/ml. v. control "t" = 0·95; P>·05			Insulin 0·5μg/ml. v. control "t" = 0·46; P>·05		
	2·5μg/ml. v. control "t" = 8·30; P<·001			2·5μg/ml. v. control "t" = 3·03; P<·02>·01			2·5μg/ml. v. control "t" = 2·13; P>·05		

* Each value is the mean of three replicate determinations.

† Variance Ratio (F) = $\frac{\text{Mean square between treatments}}{\text{Interaction mean square (rats} \times \text{treatments)}}$

TABLE 2.

Lactic acid produced by mammary gland slices (μg./100 mg. moist weight tissue/120 minutes).

Rat No.	Normal Lactating			Hypophysectomized 24 hrs.			Hypophysectomized 48 hrs.		
	Control	Insulin 0·5 μg./ml.	Insulin 2·5 μg./ml.	Control	Insulin 0·5 μg./ml.	Insulin 2·5 μg./ml.	Control	Insulin 0·5 μg./ml.	Insulin 2·5 μg./ml.
1	7*	7	65	470	438	520	398	513	427
2	35	38	218	537	445	605	402	528	472
3	42	118	147	338	398	452	265	313	310
4	78	72	133	468	487	523	377	278	300
5	110	102	163	495	545	518	302	360	333
Means	54	67	145	462	463	524	349	399	368
Variance Ratio†	F = 9·24; P<·01>·001			F = 3·90; P>·05			F = 1·46; P>·05		
"t" tests	Insulin 0·5μg/ml. v. control "t" = 0·58; P>·05 2·5μg/ml. v. control "t" = 4·05; P<·01>·001			Insulin 0·5μg/ml. v. control "t" = ·04; P>·05 2·5μg/ml. v. control "t" = 2·45; P<·05>·01			Insulin 0·5μg/ml. v. control "t" = 1·70; P>·05 2·5μg/ml. v. control "t" = ·067; P>·05		

* Each value is the mean of three replicate observations.

† Variance Ratio (F) = $\frac{\text{Mean square between treatments}}{\text{Interaction mean square (rats} \times \text{treatments)}}$

TABLE 3.

Composite respiration by mammary gland slices (µl./100 mg. moist weight tissue/120 minutes).

Rat No.	Normal Lactating			Hypophysectomized 24 hrs.			Hypophysectomized 48 hrs.		
	Control	Insulin		Control	Insulin		Control	Insulin	
		0·5 µg./ml.	2·5 µg./ml.		0·5 µg./ml.	2·5µg./ml.		0·5 µg./ml.	2·5 µg./ml.
1	133*	158	304	84	88	106	58	74	75
2	143	201	325	69	66	81	81	90	97
3	207	238	378	58	76	76	75	92	91
4	147	169	219	96	94	103	66	61	66
5	257	314	396	102	104	103	36	43	58
Means	177	216	324	82	86	96	63	72	77
Variance Ratio†	$F = 42{\cdot}95$; $P < {\cdot}001$			$F = 5{\cdot}24$; $P < {\cdot}05 > {\cdot}01$			$F = 8{\cdot}74$; $P < {\cdot}01 > {\cdot}001$		
"t" tests	Insulin 0·5µg/ml. v. control "t" = 2·36; $P < {\cdot}05 > {\cdot}01$ 2·5µg/ml. v. control "t" = 8·95; $P < {\cdot}001$			Insulin 0·5µg/ml. v. control "t" = 0·99; $P > {\cdot}05$ 2·5µg/ml. v. control "t" = 3·15; $P < {\cdot}02 > {\cdot}01$			Insulin 0·5µg/ml. v. control "t" = 2·60; $P < {\cdot}05 > {\cdot}01$ 2·5µg/ml. v. control "t" = 4·17; $P < {\cdot}01 > {\cdot}001$		

* Each value is the mean of three replicate observations.

† Variance Ratio (F) = $\dfrac{\text{Mean square between treatments}}{\text{Interaction mean square (rats} \times \text{treatments)}}$

DISCUSSION.

The responses of normal lactating rat mammary gland slices to insulin were similar to those previously described (Balmain, Cox, Folley and McNaught). The higher concentration of insulin caused a very definite increase in lactic acid production which was most likely a result of the inability of the tissue to cope, *via* the tricarboxylic acid cycle, with the greatly increased glucose uptake. With the number of replicate observations made, it is apparent that the lower concentration of insulin used gives a near minimal but significant effect on normal lactating mammary gland slices, in agreement with the observations made by Balmain, Cox, Folley and McNaught (1954). Thus small changes in sensitivity of such slices to insulin should be reflected in the response to this lower concentration of insulin. Gross decreases in sensitivity would be apparent in the response to the higher concentration of insulin.

The decreased uptake of glucose and increased lactic acid production by the control slices after hypophysectomy of the rat is confirmatory of the previous observations of Bradley and Cowie (1956). Despite the large decrease in the ability of the control tissue to take up glucose from the incubation medium 24 hours after hypophysectomy insulin is still capable of moving glucose into the cells for subsequent glycolysis. This is shown by the response of the tissue to the higher insulin concentration, although there is a decrease in sensitivity as shown by the lack of response to the lower concentration. This glucose uptake mechanism appears to be further inactivated 48 hours after hypophysectomy, although there is no substantial difference in the glucose uptake shown by control slices compared with those taken 24 hours after hypophysectomy.

Three mechanisms appear to be worth consideration at the present time with regard to control of the carbohydrate metabolism of mammary gland: (1) that a pituitary factor is normally released during lactation which controls the uptake of glucose by acting directly on mammary tissue; (2) that a pituitary factor controls the uptake of glucose *via* another endocrine gland; and (3) that a directly acting pituitary factor facilitates the action of insulin upon mammary gland glucose uptake.

There is no direct positive evidence in favour of (1) but consideration of the facts: (a) that increased metabolism of carbohydrate occurs rapidly with the onset of lactation (Folley and French, 1949), which is known to be dependent on pituitary activity (Pencharz and Long, 1933; Selye, Collip and Thompson, 1933, 1934); and (b) that intraduct injection of prolactin into a sector of mammary gland in a pseudopregnant rabbit gives rise to copious formation of milk locally (Lyons, 1942) with, presumably, increased carbohydrate metabolism by that sector, would suggest the direct action of a pituitary factor on mammary gland metabolism.

However, recent work by Randle (1956) indicates that, in the rat, removal of the pituitary may result in decreased levels of plasma "insulin" activity. If

this is substantiated it may be considered that hypophysectomy deprives the mammary gland of a level of insulin (whether this occurs by decreased secretion rate or increased utilization in other tissues) necessary to stimulate glucose uptake in this tissue. Thus one would have to postulate that a decreased plasma insulin level for part or the whole of 48 hours leads to degeneration of a mechanism which is normally responsive to insulin *in vitro*. Adrenal cortical activity is known to play a rôle in the maintenance of lactation (see Folley, 1956); however, bilateral adrenalectomy does not produce such immediate and drastic effects on lactation as measured by either the pup weight index (Cowie and Folley, 1948; Flux, 1955) or by the *in vitro* metabolism of mammary gland slices from such animals (Bradley and Cowie, 1956).

Of the third proposition no direct evidence is available, but it is known that mammary gland tissue from pregnant rats is not sensitive to insulin (Balmain and Folley, 1951). Hypophysectomy results in changes in the metabolic activity of mammary tissue which simulate a return to the type of metabolism seen in late pregnancy, *viz.*, decrease of respiratory quotient from 1·36 to 0·79 (Bradley and Cowie, 1956), under which conditions insulin is no longer effective as shown in this work. This constitutes an argument for the *in vitro* action of insulin on mammary tissue being facilitated by the presence of a pituitary factor.

Certainly the relationships between pituitary activity and response of mammary gland tissue to insulin *in vitro* are different from those shown with diaphragm muscle which responds to hypophysectomy by showing an increased *in vitro* glucose uptake and remains sensitive to insulin (Krahl and Park, 1948; Perlmutter and Greep, 1948; Bornstein and Nelson, 1948).

SUMMARY.

Hypophysectomy of lactating rats resulted in severely depressed *in vitro* glucose uptake by mammary gland slices. The glucose taken up by the slices appeared as lactic acid in the incubation medium. A decrease in sensitivity to insulin occurred within 24 hours; at 48 hours after the operation, insulin was virtually ineffective with respect to glucose uptake by such slices.

REFERENCES.

Balmain, J. H., Cox, C. P., Folley, S. J., and McNaught, M. (1954): J. Endocrinol., 11, p. 269.
Balmain, J. H., and Folley, S. J. (1951): Biochem. J., 49, p. 663.
Balmain, J. H., Folley, S. J., and Glascock, R. F. (1954): *Ibid.*, 56, p. 234.
Balmain, J. H., French, T. H., and Folley, S. J. (1950): Nature, 165, p. 807.
Barker, S. B., and Summerson, W. H. (1941): J. biol. Chem., 138, p. 535.
Bornstein, J., and Nelson, J. F. (1948): Nature, 162, p. 572.
Bradley, T. R., and Cowie, A. T. (1956): J. Endocrinol., 14, p. 8.
Cowie, A. T., and Folley, S. J. (1948): *Ibid.*, 5, p. 282.
Elliott, K. A. C., Greig, M. E., and Benoy, M. P. (1937): Biochem. J., 31, p. 1003.
Flux, D. S. (1955): J. Endocrinol., 12, p. 57.
Folley, S. J., and French, T. H. (1949): Biochem. J., 45, p. 270.

Goulden, C. H. (1952): Methods in statistical Analysis, Wiley, New York.
Krahl, M. E., and Park, C. R. (1948): J. biol. Chem., 174, p. 939.
Krebs, H. A., and Henseleit, J. (1932): Z. physiol. Chem., 210, p. 33.
Lyons, W. R. (1942): Proc. Soc. exp. Biol., 51, p. 308.
Morris, D. L. (1948): Science, 107, p. 254.
Pencharz, R. I., and Long, J. A. (1933): Amer. J. Anat., 53, p. 117.
Perlmutter, M., and Greep, R. O. (1948): J. biol. Chem., 174, p. 915.
Prohkovnik, J., and Nelson, J. F. (1953): Austral. J. exp. Biol., 31, p. 279.
Randle, P. J. (1956): Ciba Foundation Colloquia on Endocrinology, 9, p. 35.
Seifter, S., Dayton, S., Novic, B., and Muntwyler, E. (1950): Arch. Biochem., 25, p. 191.
Selye, H., Collip, J. B., and Thompson, D. L. (1933): Proc. Soc. exp. Biol., 31, p. 82.
Selye, H., Collip, J. B., and Thompson, D. L. (1934): Endocrinol., 18, p. 237.
Stadie, W. C., and Riggs, B. C. (1944): J. biol. Chem., 154, p. 687.